The regional specification of the Xenopus endoderm

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The Regional Specification of The *Xenopus* Endoderm

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Abstract

During the early stages of development the *Xenopus* endoderm becomes specified such that separate regions become committed to form different tissue types. We aimed to further understand this process, especially the role of signals from the mesoderm.

Using *in situ* hybridisation the expression patterns of 11 growth factors (FGF2, 4, 6, 8, 9, 10; Wnt3A, 5A, 7A, 8; and BMP4) were determined at tailbud stages.

Using isolated endoderm explants from stage 20-23 embryos a screen was conducted with panels of growth factors and inhibitors to try and identify the mesodermal signals. It was found that none of the factors or inhibitors was able to respecify the endoderm. These negative results indicate either that some combination of factors is responsible or that the signals may be completely novel.

Recombination experiments were carried out between endoderm and mesoderm explants. It was found that mesoderm is needed to maintain endodermal specification but that there was no reproducible instructive induction. It was also found that early stage endoderm explants are normally contaminated by mesoderm.

One source of anterior-posterior information in the mesoderm is the pattern of *Hox* gene expression. It is shown that *xHoxD13* is expressed exclusively in the mesoderm and *xHoxA13* is in both mesoderm and endoderm. *xHoxD13* overexpression does not result in any change of the endoderm, but *xHoxA13* overexpression produces a deformed gut which is flat and fails to coil. These embryos have a large persistent cavity inside the endodermal mass. However, the normal regional specification in terms of expression of *XlHbox8* and *xcad2* is still maintained. These results indicate that the abnormality arises from a defect in cell movement during gut elongation. RNA injections at the 32 cell stage showed that overexpression is necesary in both mesoderm and endoderm to obtain the defect.

I. Introduction

I.1. The Endoderm and its derivatives

Early in animal development gastrulation movements result in the formation of three principal germ layers: endoderm, mesoderm and the ectoderm. The inner most of these germ layers, the endoderm is responsible for the formation of the epithelium of two tubes within the body: the digestive tube and the respiratory tube. The digestive tube extends throughout the whole length of the body and buds from this tube form the liver, gallbladder and pancreas. The second tube, the respiratory tube, forms as an outgrowth of the digestive tube and eventually bifurcates into two lungs.

Both these tubes share a common chamber in the anterior region of the embryo, the pharynx. During development the embryo produces four pairs of pharyngeal pouches. The region between pairs of the pouches are known as pharyngeal arches. The first pair of pharyngeal pouches develops to form auditory cavities of the middle ear and the eustachian tubes. The second pair forms the walls of the tonsils in mammals. The third pair of pharyngeal pouches will contribute to the thymus. This will direct the differentiation of T lymphocytes later in development. In mouse the third pair of pouches are also responsible for the formation of the parathyroid glands. In chick however both the third and the fourth pharyngeal pouch contribute to a pair of parathyroid glands each. In addition to these paired pouches, a small, central diverticulum is formed between the second pharyngeal pouches on the floor of the pharynx. This pocket of endoderm and mesenchyme will bud off from the pharynx and migrate down the neck to become the thyroid gland (Gilbert, 2000; Slack, 2005).

Cells of the endoderm give rise only to the lining of the digestive tube whilst mesenchyme cells from the mesoderm surrounds this tube to provide muscles necessary for peristalsis. Evaginations from this tube grow, branch

and will eventually form differentiated organs such as liver, pancreas and gallbladder (Wells and Melton, 1999). The liver and gallbladder arise from the hepatic diverticulum which is a tube that extends out from the foregut into the surrounding mesenchyme. The mesenchyme induces this endoderm to branch and form the glandular epithelium of the liver. A portion of hepatic diverticulum that is closest to the digestive tube functions as the drainage duct of the liver, and a branch from this duct produces the gallbladder. The pancreas develops from the fusion of two distinct dorsal and ventral diverticula. These arise from the endoderm immediately posterior to the stomach and as they develop, they move closer together and eventually fuse. The pancreas maintains its connection to the gut through a duct similar to that used by the liver and the gallbladder (Gilbert, 2000; Slack, 2005).

The second tube from the endoderm, the respiratory tube is actually a derivative of the digestive tube. Formation of the respiratory tube begins with the laryngotracheal groove in the floor of the pharynx. This groove then goes on to develop into a separate tube to form the larynx and the trachea. The posterior end of the trachea eventually bifurcates into two branches that form the paired bronchi and lungs. In the respiratory tube, the endoderm forms the lining of the trachea, the two bronchi and the air sacs (alveoli) of the lungs (Gilbert, 2000; Slack, 2005).

I.2. Overview of Xenopus endoderm development

The development of the mature gut and its associated glands from the endoderm germ layer can be divided into three distinct steps: formation, regional specification, and differentiation (Fig 1.1). Details on each of these steps of development in *Xenopus* is outlined below.

I.2.1. Formation

In *Xenopus*, the definitive endoderm arises from cells localised to the vegetal hemisphere of the early embryo (Dale and Slack, 1987). The commitment to endodermal cell fate occurs early in development as a result

of the localisation of the maternal determinant *VegT* (Clements et al., 1999; Dale, 1999; Xanthos et al., 2001; Yasuo and Lemaire, 1999; Zhang et al., 1998). *VegT* then activates expression of *Mixer, Sox17α/ß, Xnr-1* and *Xnr-2* which will in turn activate expression of endodermal genes (Henry and Melton, 1998; Hudson et al., 1997; Jones et al., 1995). This stage of development is thought to finish by the onset of gastrulation, after the first twelve hours of development (Wylie et al., 1987). Formation of the endodermal germ layer in *Xenopus* is indicated by the expression of the pan-endodermal marker *Endodermin (Edd)* (Sasai et al., 1996).

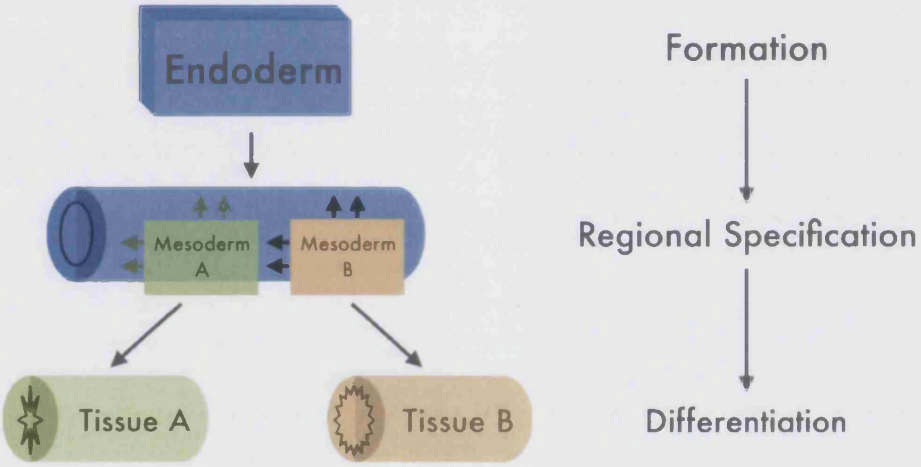

Figure 1.1 The three stages of endoderm development
Adapted from (Horb and Slack, 2001). 1) Formation. Establishment of the endodermal cell fate. 2) Specification. Patterning of the endoderm along the A-P axis. 3) Differentiation. Expression of tissue specific gene as a consequence of the patterning during specification.

I.2.2. Regional Specification

The next stage of development involves the patterning of the endoderm. Here cells of the endoderm are given positional information along the anterior-posterior (A-P), dorsal-ventral and left-right axes. These patterning will in turn allow the endoderm to form the many different specialised tissue types found in the fully developed gut. In short Regional specification of the endoderm is the commitment of each tissue region, which is manifested in culture in a neutral medium but may still be reversible if the environment changes (Slack, 1991a). Patterning of the endoderm at

this stage of development is usually marked by the expression of *Xlhbox8* in the anterior part (Wright et al., 1989) and *Xcad2* (van den Akker et al., 2002) in posterior endoderm. More detailed discussion of this part of endoderm development can be seen in Section 1.6.1 (p20). There may also be advanced activation of some differentiation products such as *IFABP* during this stage of development (Shi and Hayes, 1994).

I.2.3. Differentiation

The final stage in the development of the endoderm involves the differentiation of the gut based on the patterning information received by the cells during regional specification. This usually involves the expression of tissue specific genes (e.g. *IFABP, LFABP*, and insulin) (Horb and Slack, 2001). Expression of these terminal differentiation markers is indicative of the formation of the mature digestive tract and its associated organs (Grapin-Botton and Melton, 2000). This process is thought to occur sometime after day 3 of development (~stage 40) in *Xenopus* (Horb and Slack, 2001).

I.3. Regional specification in *Cynops (Triturus) pyrrhogaster*

One of the earliest studies regarding endoderm patterning was done in newt, *Cynops* (previously *Triturus) pyrrhogaster*. The study relied on endoderm and mesoderm explants generated from blastula and neurula stage embryos. These tissue explants were then recombined and left to develop in culture. The identity of the explant was then characterised using histological techniques. Here it was demonstrated that the endoderm explants from early gastrula which are free from mesoderm and left to grow on its own stay as 'yolk mass' and will not differentiate. However when the endoderm explants were made with significant quantities of mesenchyme, they went on to develop differentiated endodermal organs. This then indicated an important role the mesoderm plays in endoderm development (Fig 1.2A) (Okada, 1954a; Okada, 1954b; Okada, 1955a; Okada, 1955b).

Further recombinations have also shown that the type of mesenchyme has an influence on the type of tissue the endoderm would differentiate into (Okada, 1955a; Okada, 1955b; Okada, 1957; Okada, 1960). Endoderm that is normally fated to form the anterior structure such as pharynx was shown to only produce posterior organs such as intestine when combined with lateral mesoderm (Fig 1.2B). Similarly anterior and middle endoderm explants were found to only form pharynx when put near head-mesenchyme. These recombinations then showed that the mesoderm is capable of instructing the endodermal fate.

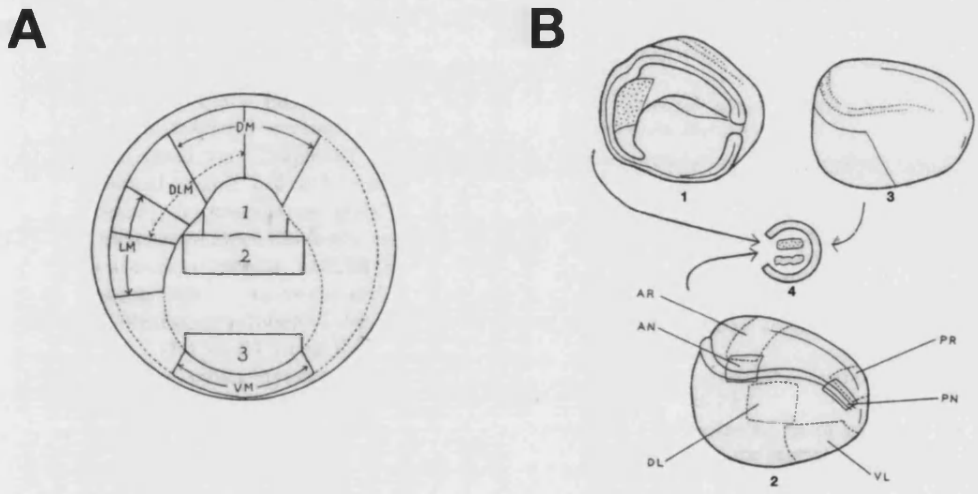

Figure 1.2 Newt recombinations studies.
A) Taken from (Okada, 1954a) Ventral view of the early gastrula showing the positions of the explanted pieces. 1. Anterior endoderm, 2. Middle endoderm 3. Posterior endoderm. The mesodermal tissue added to the endodermal pieces are taken from dorsal (DM), lateral (LM), dorso-lateral (DLM) or ventral (VM) part of the marginal mesoderm. B). Taken from (Okada, 1957) Scheme of recombination experiment. (1) shows location of pharyngial primordium source. (2) shows source anterior and posterior neural plate (AN and PN), anterior and posterior archenteric roof (AR and PR) and dorsal and ventral lateral plate (DL and VL). (3) shows isolation of enveloping epidermis. These were removed from the embryo and recombined as in (4).

However these early studies carry several important limitations. At the time these recombinations was performed, a detailed fate map had not yet been established. This was mainly due to a lack of available specific lineage tracers at the time. A good fate map is important when studying specification of the endoderm and mesoderm as it allows for the

comparison of experimental results with the presumptive fate for that particular piece of tissue. The lack of a lineage tracer also meant that it was impossible to eliminate the possibility of contaminating cells in the recombinations as it would have been impossible to label the different tissues in the recombination. In other words it was impossible to conclusively determine whether the anterior endoderm explant does not contain any posterior contamination or vice versa. This also raises the possibility that the respecified endoderm observed in the experiment was a result of contaminating endoderm that was isolated along with the mesoderm.

At the time there was also a lack of molecular markers that label the different parts of the developing gut. Even though it is true that the different parts of the gut could be identified histologically, molecular markers would have allowed for a more accurate detection of the different tissue types, thus allowing for better detection of respecification in the endoderm. Also molecular markers for either endoderm and mesoderm would have allowed for detection of any contaminating cells in the explants, allowing for a more conclusive interpretation of the data.

I.4. Regional specification in mouse

I.4.1. Recombination studies

Role of epithelial-mesenchymal interactions during endoderm development in mouse was initially studied using recombination of the forestomach and glandular stomach in fetal mice. Here it was demonstrated that the epithelium's survival depends on the presence of mesenchyme. Interestingly, heterologous recombination did not change the fate of the epithelium. Forestomach epithelium was not respecified when combined with glandular stomach mesenchyme and the opposite is also true: glandular stomach epithelium maintains its differential fate when recombined with forestomach mesenchyme. However it is worth noting that the type of mesenchyme present still has an effect on the rate of development of the endoderm. The rate of keratinization of the forestomach

epithelium was found to be greater on recombination with homologous mesenchyme (i.e. with forestomach mesenchyme) than on recombination with heterologous mesenchyme (e.g. glandular stomach mesenchyme) (Fukamachi et al., 1979). A similar conclusion was achieved when the experiment was duplicated in rats (Fukamachi and Takayama, 1980).

There are two ways in which the mesoderm can influence the development of the endoderm. In a permissive model the signals sent by the mesoderm enable the growth and survival of the endoderm while maintaining an existing prepattern in the endoderm; thus, the type of endodermal tissue formed would be dependent on the original position of the endodermal tissue. For example an endoderm explant isolated from the anterior of the embryo would maintain its anterior fate when recombined with posterior mesoderm. The opposite of this model is an instructive model whereby the mesoderm is defining the identity of the endoderm along the A-P axis so that the type of endodermal tissue formed is dependent on the original position of the mesodermal tissue. For example an anterior endoderm, when recombined with posterior mesoderm would take on a posterior fate. These early recombination results in mouse then seem to support a permissive model of endoderm specification as the recombined mesoderm is unable to respecify the endoderm. However these recombinations was done with quite late embryos and as such the epithelium might have lost some of its plasticity and thus can not be respecified. It is important to note that even though these recombinations give insight into the timing and stability of the specification of the endoderm it does not indicate how the endoderm gains its original specification earlier in development.

Fate mapping in mouse was done using embryos at E7.5 was done by microinjecting horseradish peroxidase (HRP) into single axial endoderm cells *in situ* (Lawson et al., 1986; Lawson et al., 1991). The labelled endoderm descendants was then traced to early somite stages *in vitro*. Here they divided the endoderm at E7.5 to 4 regions (Fig 1.3). Region I, the most

anterior endoderm, maps to the ventral foregut in somite-stage embryo (E8.5) which gives rise to liver, ventral pancreas, lungs, and stomach. Region II maps to dorsal foregut endoderm, which contributes to esophagus, stomach, dorsal pancreas, and duodenum. Region III maps to midgut/trunk endoderm, which forms the small intestine. Region IV, the most posterior endoderm, maps to posterior trunk endoderm and hindgut, which forms the large intestine.

Following from this, Wells and Melton (2000) dissected the four endodermal regions, as described by the fate map, from E7.5, E8.5 and E9.5 day mouse embryo and analysed expressions of various endodermal markers (Fig 1.3). Here they found that *Hesx1*, *ß-cardiac actin* and *cerberus-like* to be expressed in anterior endoderm (regions I and II) of E7.5 embryo whilst *IFABP* was found to be expressed in the posterior endoderm (regions III and IV). It is interesting to note that *ß-cardiac actin* was expressed mostly in endoderm and not mesoderm at this stage of development, thus making it suitable to be used as marker of endoderm development.

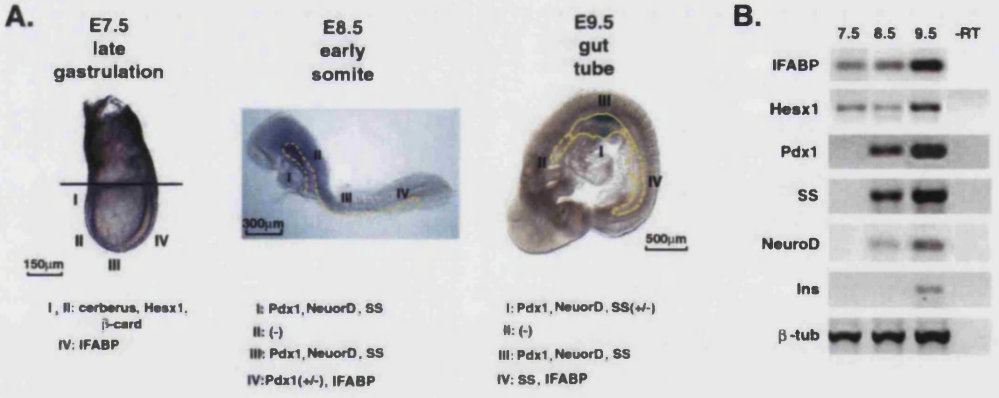

Figure 1.3 Fate map of the mouse endoderm
Taken from (Wells and Melton, 2000) and reproduced with permission of the Company of Biologists. A). Shows the four regions of the mouse endoderm and its progression between E7.5 and E9.5. Spatial expression of the markers are also laid out for the different stages of development. B) RT-PCR showing the activation of the endodermal markers between E7.5 and E9.5

Expression of the genes *Pdx-1*, *NeuroD* and *somatostatin (SS)* was observed later as the gut tube is formed (E8.5). *Pdx1* expression could be

seen as early as the 3-somite stage whilst *NeuroD* and *SS* were only detectable at 5 somite stage (Gittes and Rutter, 1992; Jonsson et al., 1994; Naya et al., 1997). By E9.5 *Pdx-1* expression was restricted to pancreatic regions I and III. *NeuroD* and *SS* were also expressed in this region however the SS expression extend to the posterior gut tube that will give rise to the intestine (Region IV) (Gittes and Rutter, 1992). The early expression of *ß-cardiac actin* and *IFABP* is interesting as it raises the possibility that endoderm from E7.5 already has positional identity indicating that the anterior and posterior most parts of the endoderm is specified earlier than the rest of the endoderm.

Indeed this hypothesis seemed to be supported with results from the endodermal explants. Here it was shown endodermal explants created from E7.5 embryo, free of the mesectoderm showed expression of the extreme anterior and posterior markers *ß-cardiac actin* and *IFABP* but not of the intermediate markers: *Pdx1, SS* and *NeuroD* (Wells and Melton, 2000). There are two possible explanation for this, the first is that the signals required for the anterior and posterior development operates prior to E7.5 when the explants was made. The second is that there is a possibility that there might be mesodermal cells present in the endoderm explant. This is because even though the explants were negative for the mesodermal marker *FGF4*, low level of *T(Bra)* expression was detected, indicating that there might be some mesodermal cells present. However, regardless of the cause for the early expression of *ß-cardiac actin* and *IFABP*, the lack of expression of the intermediate markers (*Pdx1, SS* and *NeuroD)* indicate that complete patterning of the endoderm must require the presence of adjacent mesectodermal layer.

Next the study goes on to address whether the mesectodermal signals responsible for the differentiation of endoderm are instructive or permissive. They divided the endoderm and the mesectoderm into anterior and posterior pieces (Fig 1.4) and did heterologous and homologous recombination between them. The resulting explant was analysed using four

endodermal markers *ß-cardiac actin, IFABP, SS and NeuroD*. The results from this indicated that the mesectoderm was capable of respecifying heterologous endoderm explants. A posterior endoderm explant when recombined with anterior mesectoderm had a markedly stronger *ß-cardiac actin* expression whilst showing repression of *SS* and *NeuroD*, in other words the endoderm had taken a more anterior fate. An anterior endoderm, on the other hand, when recombined with posterior mesectoderm shows a slight reduction in *ß-cardiac actin* expression and an induction of *IFABP* and *SS*, showing posterior respecification (Wells and Melton, 2000).

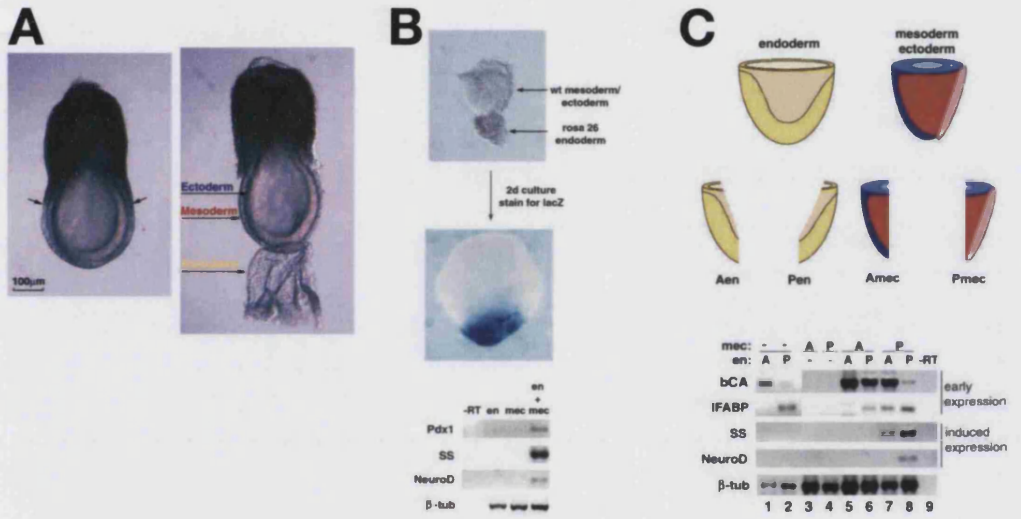

Figure 1.4 Mouse recombination experiment.
Taken from (Wells and Melton, 2000) and reproduced with permission of the Company of Biologists. A) E7.5 embryo and the three germ layers B) Isolated endoderm cultured alone or in contact with mesectoderm (top). Endoderm expressing ß-gal (isolated from rosa 26 mouse) spreads out along the mesoderm/ectoderm after 2 days of coculture (middle). Also PCR done on the cultured recombinants show expression of *Pdx1, SS and NeuroD* is maintained only in the presence of mesectoderm. C)Diagram showing how the endoderm and mesectoderm are divided to perform heterologous recombination. PCR results on this show that signals from mesectoderm are instructive.

It is interesting to note that from these heterologous recombination experiment that expression of *IFABP* and *ß-cardiac actin,* which have been shown previously to not require mesectoderm in E7.5 mouse, is now affected. This raises the question as to what is the original role of the mesectoderm in the normal development of the anterior most and posterior most part of the endoderm? The initial expression of these markers seems

to be autonomous, therefore the mesectoderm's logical role would have been to maintain these signals, in other words sending permissive signals allowing maintenance of the early patterning which is opposite to results from the heterologous recombination where the mesectoderm is acting in an instructive manner.

However we need to remember earlier that there were low levels of *T(Bra)* detected earlier in the endoderm explants. If we assume that indeed the presence of mesodermal cells was responsible for the initial expression of *IFABP* and *ß-cardiac actin* then the instructive mesectoderm in the heterologous recombination would be the consistent result.

I.4.2. Identifying mesectoderm signals

The recombination experiments have shown that the mesectoderm is able to influence the development of the endoderm in mouse. However it was not known yet whether these influences require cell-cell contact or whether it was done through soluble factors. Wells and Melton (2000) addressed this by taking isolated endoderm and mesectoderm and placing a 0.4 µm pore diameter Nucleopore membrane between them. The size of 0.4 µm is probably a compromise based on an earlier study in chick that found 0.2 µm is the size that allows passage of soluble factors whilst minimising cell-cell contact, and anything larger than 0.6 µm would allow whole cells to pass through (Takiguchi-Hayashi and Yasugi, 1990). This membrane then forms a physical barrier that prevents most cell-cell contact whilst still allowing soluble factors to pass through between the two germ layers. Hence if the signals are soluble the endoderm should still receive instructive signals from the mesectoderm layer and develop appropriately. On the other hand if the mesectoderm requires prolonged cell-cell contact to properly influence the development of the endoderm then the endoderm would have stayed as is, and not express any of the endodermal patterning markers.

After a 2-day culture, the endoderm explants showed expression of specification markers *Pdx1*, *SS*, *NeuroD* and insulin (Wells and Melton, 2000). These results then indicate that in order for the mesoderm to specify the endoderm prolonged cell-cell contact is not necessary and that the influence is most likely facilitated by soluble factors. However it is worth noting that the membranes used in these experiment inevitably reduce the signal significantly. Thus even if the signal is diffusible, the cross section available for diffusion is much reduced. This then would account for the better survival of the endodermal explants when they were in direct contact with mesectoderm.

Based on this, a screen study was done of several growth factors. In mouse they found that out of 10 growth factors tested, only FGF4 was found to elicit an effect on the endoderm. This effect was dose dependent with *NeuroD* induced at low doses (0.1-1 ng/ml) and *SS* induced at higher doses (1-10 ng/ml). (Wells and Melton, 2000). This concentration dependence implies that FGF4 acts as a posterior morphogen. Members of the *FGF* family have been known to have strong posteriorising activity which is consistent with the posterior dominant patterning of the endoderm by *FGF4.* Overexpression of *FGF4 homologue eFGF* in *Xenopus* have lead to severe posteriorisation of the embryos, indicating that it is capable of patterning the embryo in a posterior manner (Christen and Slack, 1997; Isaacs et al., 1994; Pownall et al., 1996).

in vivo, FGF4 is normally expressed in the posterior of the embryo (primitive streak) (Isaacs et al., 1992a; Isaacs et al., 1992b; Niswander and Martin, 1992). The fate map earlier showed that in normal development *SS* is expressed in more posterior endoderm, closer to the primitive streak, and as such closer to *FGF4*. Thus it is not surprising to see that a higher dose of FGF4 is needed to induce *SS* expression in isolated endoderm. Consistent with this, *NeuroD* which is expressed more anteriorly, relative to *SS*, responds to lower doses of FGF4 in the explant. In fact a high concentration of FGF4 (10ng/ml) was shown to repress *NeuroD* expression in the

endodermal explant (Wells and Melton, 2000). These is consistent to the posterior morphogen model where different thresholds of *FGF4* expression leads to the development of distinct endodermal structure.

It is interesting to note that in this particular screen out of the FGFs tested (aFGF, FGF2, 4, 5, 8) only FGF4 elicited a response. A possible reason for this is that the FGF receptors expressed in the endoderm at this time, FGFR1, has several different splice variants (Wells and Melton, 2000). Each of these variants have different response to different FGFs. A cellular reporter assay found that the FGFR1 isoform b to be threefold more responsive to FGF4 than FGF2, and tenfold more responsive to FGF4 when compared with FGF5 or FGF8 (Ornitz et al., 1996). At the moment this is just speculation as the exact variant of FGFR1 expresed in the endodermal explant has yet to be characterised. Also, sometimes biochemical data on binding specificity obtained *in vitro* often does not match with *in vivo* biological activities. Thus this preferential binding to FGF4 *in vitro* might not have any consequence on its *in vivo* activity in endoderm specification.

We need to note however that the above interpretation is a rather straight forward one. There are other interpretations that need to be considered. FGF4 in its role in patterning the endoderm might do this by first inducing mesoderm formation in the endodermal explant. This mesoderm would then in turn pattern the endoderm, in other words the FGF would elicit a secondary effect on endoderm patterning. Wells and Melton did not address this in their study as they did not screen for mesodermal markers on their FGF4 treated explants. Also we need to consider the observation of a low level expression of *T(Bra)*, a mesodermal marker, in isolated endoderm. This raises the possibility that FGF4 might instead act on the remaining mesodermal cells in the explant and not directly on the endoderm (Horb, 2000).

I.5. Regional Specification in Avian systems

I.5.1. Epithelial-mesenchymal interactions

In chick regional differences of the digestive tract are recognizable soon after the establishment of a tubular tract by the form and the position of digestive organ rudiments. Morphological and functional differentiation of these different organs is dependent on cross talk between the endodermal epithelium and the mesenchyme. (Mizuno and Yasugi, 1990; Yasugi and Mizuno, 1990).

Recombination experiments have shown that the mesenchyme is capable of instructing the endodermal fate in chick. For example the gizzard (muscular stomach) in chick is usually devoid of any glands at hatching. However when the gizzard endoderm is recombined with proventricular or intestinal mesenchyme they go on to develop proventriculus-type and intestinal-type endocrine cells respectively (Andrew and Rawdon, 1990; Andrew et al., 1988; Rawdon and Andrew, 1988). Furthermore the intestinal mesenchyme has also been shown to be capable of respecifying the stomach epithelium towards the intestinal fate (Haffen et al., 1983; Ishizuya-Oka and Mizuno, 1984; Ishizuya-Oka and Mizuno, 1992). These results then show that in avian systems, unlike in mouse, late recombinations of endoderm and mesoderm could still lead to respecification of the endoderm. This difference might be caused by a later stabilisation of patterning in avian embryos compared to murine embryos.

Fate maps for gut endoderm and mesoderm for the 10-somite chick embryo, established from DiI lineage tracing studies, show that endoderm and mesoderm that will be in contact in the gut wall of the older embryos are not perfectly aligned in early embryos. In early embryos the endoderm is always positioned slightly more anteriorly relative to its final position. As development progresses, both endoderm and mesoderm move caudally with the endoderm moving more rapidly, resulting in alignment of the related

endodermal and mesodermal regions at the beginning of gut closure (Matsushita, 1995; Matsushita, 1996). This observation has not been reported as yet in the mouse.

Since, it is thought that the patterning of the endoderm is not stabilised until the endoderm and mesoderm reach their final relative positions, one can hypothesise that If the mouse endoderm is aligned to its appropriate mesodermal regions during development, the specification of mouse endoderm might be stabilised sooner than the chick endoderm. The chick endoderm needs more time to align with its mesoderm. This then could account for the difference in plasticity between the two model organism.

I.5.2. Lateral Plate Mesoderm (LPM) and endoderm specification

Recently a more detailed analysis of the interaction of mesoderm and endoderm was done in chick. In this study the developing gut was divided into 3 distinct regions along its A-P axis (Fig1.5). Fate mapping studies show that these regions develop to form oesophagus and stomach (somite 2-4 level, block a), duodenum and ventral pancreas (somite 7-9 level, block b), and more posterior small intestine (somite 12-14 level, block c) (Kumar et al., 2003; Matsushita, 1996).

Pdx1, the pancreatic transcription factor and *CdxA*, the intestinal transcription factor are used as specification markers. They are expressed in distinct endodermal regions and can be used to distinguish the 3 regions of the endoderm effectively (Fig 1.2) (Kumar et al., 2003). Note that the genes *Hex1* and *Nkx2.1* are also expressed in region I the anterior most region (Dessimoz et al., 2006) but these are not used as markers in the study.

Using a chick/quail chimera technique, it was shown that an anterior endoderm that is grafted posteriorly towards *Pdx1*-expressing region results in the upregulation of *Pdx1* in the graft. Similarly grafting endoderm of medial domain endoderm towards the posterior domain resulted in the downregulation of *Pdx1* and upregulation of *CdxA*. Interestingly, however,

when the posterior endoderm was grafted anteriorly towards the *Pdx1*-expressing domain, expression of *CdxA* was not downregulated and no induction of Pdx1 was seen. The same was seen when medial domain endoderm was grafted towards the anterior, *Pdx1* expression was retained. This then shows that only posterior shifts of the endoderm would result in the respecification of the endoderm whilst anterior shifts maintain the original fate of the endoderm (Kumar et al., 2003).

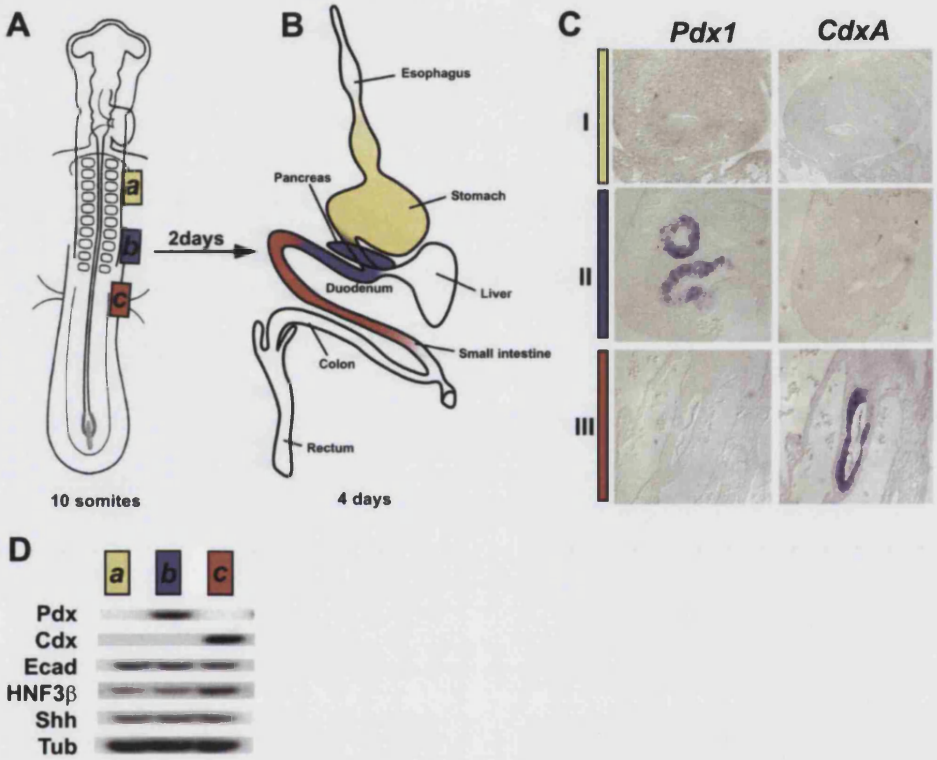

Figure 1.5 Regions of the developing chick gut.
Taken from (Kumar et al., 2003)[1] A) The diagram shows how the endoderm is divided into three different regions: foregut (a), midgut (b), hindgut (c) with the fate map shown on B). C) shows *in situ* of the three different regions for *Pdx1* and *CdxA*. *Pdx1* is shown to be present only in the midgut (b) whilst *CdxA* is only present in the hindgut (c). D) Shows the PCR for various markers of endoderm development. Expression pattern of *Pdx1* and *CdxA* confirms the *in situ* result shown in C.

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[1] Reprinted from Dev Biol, 259, Kumar et al., Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate, 109-22, Copyright (2003), with permission from Elsevier

This observation was confirmed further by heterotopic recombinations between endoderm and LPM. Similar to the *in vivo* study using chick/quail chimera, the endoderm was only respecified when combined with a more posterior mesoderm (induction of *Pdx1* in foregut by midgut LPM and induction of *CdxA* and inhibition of *Pdx1* in midgut by hindgut LPM). Recombination with anterior LPMs resulted in the maintenance of endodermal fate (Kumar et al., 2003).

The *in vitro* and *in vivo* study seem to suggest that the mesodermal signals are instructive if they come from more posterior regions to the endoderm and permissive otherwise. This is in contradiction to the status in mouse (see Section 1.4.1, p6) or even other studies in chick where the respecification occurs not just posteriorly but also anteriorly (Ishii et al., 1997; Ishii et al., 1998; Wells and Melton, 2000). In the chick, using tissue from day 6 embryos, after organ formation and cytodifferentiation, posterior epithelium from the small intestine was shown to adopt an anterior (stomach) gene expression when cultured with the anterior mesenchyme (Ishii et al., 1997; Ishii et al., 1998). It is important to note however that the culture period done with the small intestine and stomach recombination was much longer (6-8 days) than the culture with endoderm and LPM (48 hours). Also the tissue used in the recombination was from different developmental stages, as such, the fact that anterior respecification can be induced from later stage tissue might just indicate changes in gene regulation at later stages in development (Ishii et al., 1997; Ishii et al., 1998; Kumar et al., 2003).

Another explanation for the posterior-dominant patterning of the endoderm by the mesoderm involves the nature of the markers used. *Pdx1* and *CdxA* are members of the *ParaHox* cluster of transcription factors and like other members of the *homeobox* family they might be susceptible to a cluster-wide regulation. However since the LPM also regulates other pancreas markers outside of the *ParaHox* cluster such as *glucagons, insulin Nkx6.1* and *p48*, this is unlikely to be the case (Kumar et al., 2003).

The posterior dominance observed in the explants could also be a consequence of a posterior gradient of morphogen. In this case, transient exposure to a higher level of morphogen (moved posteriorly) can induce a higher response from the tissue if it remains competent. However the opposite is not true, when exposed to a lower amount of morphogen (moved anteriorly) the response from the tissue does not change, as the higher response state that have been induced are already stable.

Regardless of the mechanism, the posterior-dominant patterning of the endoderm shown here is strikingly similar to observations in the neural tube at the same stage. Neuroectodermal segments have been shown to only respecify when they are transplanted to a more posterior region where as anterior transplantation just results in maintenance of the original expression profile (Grapin-Botton et al., 1995).

I.5.3. Identifying signals from LPM

Attempts have been made to understand the molecular basis of the mesodermal signals in chick embryos. The first issue that was addressed was whether cell-cell contact is necessary for the mesoderm to instruct the endoderm. To address this a transfilter analysis was performed. Here endoderm and mesoderm was separated by a Nucleopore filter with a pore size between 0.2 μm and 0.6 μm. Here they found that mesoderm (proventricular mesenchyme) can induce specification in the proventricular and gizzard epithelia) indicated by gland formation and pepsinogen expression when they were separated by pores of 0.6μm and not 0.2μm (Takiguchi-Hayashi and Yasugi, 1990). This finding then suggests that soluble factors from the mesoderm might not be enough to respecify the endoderm and that direct contact with mesoderm cells is probably necessary for specification of the endoderm.

This was supported with results from a recent growth factor screen done on chick endodermal explants (Kumar et al., 2003). Here they found that the growth factors would not elicit an effect on the endoderm without

the presence of mesoderm. They showed that Retinoic acid (RA), BMP and activin family members can induce posterior transformation with endoderm+ mesoderm explants but not with isolated endoderm from the same region. The authors noted however that this does not mean that the growth factors do not act directly on the endoderm. Instead it was suggested that the mesoderm is necessary to provide the additional factors needed to elicit a response. In other words the specification is not done by a single growth factor and needs a combination of factors to act at once to elicit a response (Kumar et al., 2003).

FGF4 was also later discovered to be involved in patterning the endoderm on chick embryos. Heparin agarose beads that have been soaked in FGF4, and subsequently implanted into gastrulation stage chick embryo, was capable of inducing more posterior fate on the endoderm. In the anterior most endoderm FGF4 was seen to repress expression in more anterior markers of development (*Hex1* and *Nkx2.1*) whilst promoting expression of more posterior markers (*Pdx1* and *CdxA*). An inhibition study using heparin agarose beads soaked in the chemical SU5402, a FGFR1 inhibitor, confirmed the role of FGF4 in the patterning of the endoderm. Here inhibition of FGF signaling resulted in expansion of the expression of the anterior most marker *Hex1* towards the position of the beads as well as repression of more posterior fate *Pdx1*. At early somite stages implantation of FGF4 beads caused expression of *Pdx1* in the anterior most endoderm. Reduction of FGF4 signalling at these stages by SU5402 resulted in *Pdx1* expression shifting from fore/midgut in normal embryos to the mid/hindgut and repression of *CdxA* in mid/hindgut (Dessimoz et al., 2006).

The study went on to show that when dominant negative FGFR1 receptor, when electroporated onto an embryo, ectopic *Pdx1* expression was induced. This demonstrates that the endoderm is directly affected by the FGF signaling (Dessimoz et al., 2006). However because the experiment was done on whole embryos with all three germ layers present and not isolated endoderm, one cannot exclude the possibility that the mesoderm

was involved in the specification of the endoderm by FGF4. The mesoderm around the endoderm might contribute signals that cooperate with the FGF4 signal in specifying the endoderm. Hence, in the case of chick embryos it seems that there is still a possibility that contact with mesoderm is necessary for the specification of the endoderm.

The discovery that soluble growth factors can replace or at least mimic the signals coming from the mesoderm and pattern the endoderm is a significant one (Dessimoz et al., 2006; Kumar et al., 2003; Wells and Melton, 2000). FGF4 is particularly adept in patterning the endoderm as it has been shown to posteriorise the endoderm in both mouse and chick(Dessimoz et al., 2006; Wells and Melton, 2000). This raises the possibility that the regional specification of endoderm might be controlled by a posterior gradient of FGF4 to form a rough patterning of the endoderm which is refined later in development by additional permissive signals from the mesoderm.

I.6. Regional specification in *Xenopus*

I.6.1. Markers of specification in *Xenopus*

Four endodermal markers have been used extensively in the study of endoderm development in *Xenopus*: *Xlhbox8, IFABP, Xcad2* and *Edd*. It is necessary to spend a bit of time to understand how these markers came to be used and why they are appropriate markers to study endoderm development.

As we have seen, in the discussion regarding regional specification in mouse and avian systems, that anterior specification is usually marked by the expression of the pancreatic development marker *Pdx1*. The *Xenopus* orthologue of this is *Xlhbox8*. First isolated in 1988, it was found to be expressed solely in a narrow band of the endoderm in early *Xenopus* embryos. In later stages the expression was restricted to endodermal cells of the duodenum and the developing pancreas, tissues originating from anterior endoderm (Chalmers and Slack, 2000). Expression of the RNA has been detected as early as stage 12.5 with more expression detected as the

embryo grows. The protein itself, however is not expressed until stage 33 (Wright et al., 1988). The restricted expression of *Xlhbox8* to anterior endoderm structures has made it an ideal marker of anterior development in *Xenopus*.

The situation with the posterior marker of development however is more complicated. Earlier study of specification in the endoderm had used *IFABP* (Gamer and Wright, 1995; Henry et al., 1996; Shi and Hayes, 1994; Zorn et al., 1999) instead of *Xcad2* as a posterior marker. However a recent study in our lab has shown that *IFABP* is more appropriately used as an intermediate marker as it is found to be derived from both anterior and posterior halves of the neurula endoderm (Chalmers and Slack, 2000) which would account for *IFABP* expression in both the dorsal and ventral vegetal explants (Henry et al., 1996). This has been confirmed with further *in situ* and RT-PCR data (Horb and Slack, 2001). Furthermore *IFABP* is a product of differentiation that is expressed early and not a transcription factor as is the case for *Xcad2*. Since regional specification occurs prior to differentiation, a marker which is a product of differentiation such as *IFABP* would not be appropriate. *Xcad2* which is a transcription factor would be the more appropriate posterior marker to use in studying the specification of the endoderm (Horb and Slack, 2001). This is indeed a trend we see now in mammalian studies as well, with the more recent studies using *Cdx2* and not *IFABP* to mark posterior development (Dessimoz et al., 2006; Kumar et al., 2003). We believe it is better to use transcription factors to mark regional specification as they are more likely to be involved in the specification process itself and not a consequence of it. However it is worth noting that this does not reduce the validity of the earlier studies that used *IFABP* instead of *Xcad2* as posterior markers of specification since the expression of the gene itself still proves that there is specification in the vegetal explants.

Finally there is the pan-endodermal marker gene *Endodermin (Edd)*. This gene encodes a large protein with significant homology to α2-M family

protease inhibitors. Initial expression of *Edd* is found in the dorsal lip during gastrulation, which then spreads to the entire rim of the blastopore. Histological sections at this stage shows *Edd* transcripts to be strongest in cells fated to form the endoderm, even though it is also found in axial mesoderm precursors. In late neurula it is found to be expressed in the notochord, prechordal plate, hatching gland and the whole endoderm. Its expression then becomes even stronger at tailbud stages where it is localised almost exclusively to the endoderm (Sasai et al., 1996). The abundant expression of the *Edd* gene in endodermal cells from early gastrula makes it ideal to be used as a pan-endodermal marker in *Xenopus*.

I.6.2. Early or Late?

Until recently much of the work done in *Xenopus* have suggested the specification of the endoderm might have taken place early in development, prior to gastrulation and cell-autonomously in the absence of mesoderm. This model for endoderm specification first came about in a study of *Xlhbox8* regulation. It was found that vegetal explants made from stage 8-9 blastula embryo (Fig 1.6) would go on to express *Xlhbox8* in culture. The gene was found to be expressed in a contiguous domain of cells occupying about a quarter to a third of the explant with a similar time course of expression as in whole embryos. This expression of *Xlhbox8* is attributed to the dorsal half of the vegetal explant (Gamer and Wright, 1995). A later study confirms the autonomous expression of *Xlhbox8* in dorsal vegetal explant. The study also found both posterior markers *IFABP* and *insulin* to be expressed from the vegetal explants. This then further supports an early and autonomous model of endoderm specification in *Xenopus* (Henry et al., 1996). Anterior endoderm was shown to be specified as early as blastula stages as marked by the expression of *Xhex* and *Cerberus* (Zorn et al., 1999).

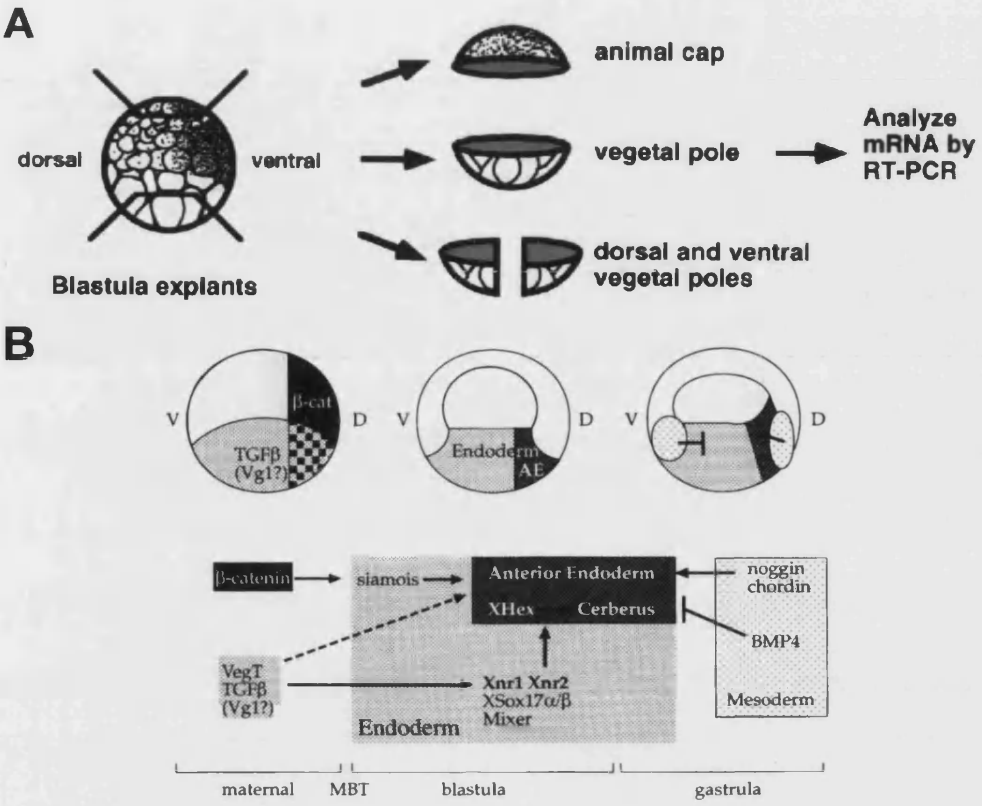

Figure 1.6 Vegetal explants
A) Taken from (Henry et al., 1996) and reproduced with permission of the Company of Biologist. Generation of the vegetal explants. The diagram above showed how the dorsal and vegetal explants was generated from blastula stage embryo. The subsequent explants was then grown in culture and analysed for specification markers by RT-PCR. B) Taken from (Zorn et al, 1999)[2]. Diagram for the mechanism of Anterior endomesoderm development. The top diagram shows the spatial positioning of the signals and the bottom diagram shows the signalling cascade involved in the autonomous specification of the endoderm.

This early and autonomous pattern of the endoderm in *Xenopus* is thought to involve events and molecules that are also important in the induction and patterning of the mesoderm. The specification of anterior endoderm specifically has been shown to start before gastrulation as *Xhex* and *Cerberus* were expressed before the gastrulation stage around the

[2] Reprinted from Dev. Biol, 209, Zorn et al., Anterior endodemesoderm specification in Xenopus by Wnt/beta catenin and TGF-beta signaling pathways, 282-97, Copyright (1999), with permission from Elsevier.

same time that the Spemann organiser is induced. The specification of the anterior endoderm seems to involve two maternal signalling pathways: the dorsalising Wnt/ß-catenin pathway as well as an endodermal-specific TGF-ß signalling pathway (Henry et al., 1996; Zorn et al., 1999). These two pathways then initiate a signalling cascade which are summarised in Figure 1.6B. Expression of *Xlhbox8* has also been shown to rely on *TGF-ß* as well as on correct cortical rotation of the embryo as inhibition of cortical rotation leads to loss of *Xlhbox8* expression (Henry et al., 1996).

Posterior endoderm specification however is less explored. Expression of *IFABP* in vegetal explants did not seem to be affected by either truncated activin or dominant negative FGF receptors. Also, unlike *Xlhbox8* it is not affected by inhibition of cortical rotation. It is however downregulated in UV treated embryos (Henry et al., 1996). The downregulation in the UV treated embryos are thought to be caused by an excess of ventrolateral mesoderm that acts negatively on IFABP expression and not as a result of disruption to cortical rotation.

These results seemed conclusive at the time, however one must remember that the endoderm and mesoderm are still closely related at the blastula stage. As such there is a risk of mesoderm contamination in the explant, which in turn might cause the expression of the specification markers. To ensure that this was not the case these studies screened the vegetal explants with the mesodermal marker *Brachyury (Xbra), cardiac actin, Xtwist and* α*-T*$_3$ *globin* (Gamer and Wright, 1995; Henry et al., 1996; Zorn et al., 1999). However, these markers might not be appropriate to detect gut mesoderm that could be present in the vegetal explants. *Xbra* is only found to be expressed in the tail bud at early tadpole stages (Gont et al., 1993; Smith et al., 1991), *muscle specific actin* is localised to the somatic and heart mesoderm, *type II collagen* is localised to the notochord (Amaya et al., 1993), *Xtwist* is expressed in the neural crest (Hopwood et al., 1989; Schuh et al., 1993) and α*-T*$_3$ *globin* is expressed in the ventral mesoderm at tail bud and early tadpole stages making it unlikely to be

found in the mesoderm surrounding the gut at late tadpole stages (Horb and Slack, 2001).

Indeed using mesodermal markers that are found in the gut mesoderm such as *FoxF1* (Koster et al., 1999) and *XNkx-2.5* (Evans et al., 1995; Patterson et al., 2000; Smith et al., 2000) as well as those found in lateral plate, *XTbx5* (Horb and Thomsen, 1999) and *xFOG* (Deconinck et al., 2000), Horb and Slack (2001) were able to show the presence of mesoderm in the vegetal explants. This discovery meant that the apparent cell autonomous specification of the endoderm might have been caused by patterning done by the presence of mesodermal cells. There are two possibilities as to where these mesodermal cells came from: the first is that they might have been a result of incomplete separation done during the generation of these vegetal explants.

The second possibility is that these mesoderm cells might have been formed de novo in the vegetal explants, as a response to being cultured *in vitro*. Under normal development inducing signals from the endoderm form a morphogen gradient that instructs cells adjacent to the endoderm to take on a mesodermal fate and those furthest away to take on a ectodermal fate (Slack, 1991a). Isolated vegetal explants when recombined with animal caps are able to convert the animal cells towards a mesodermal fate (Nieuwkoop, 1969; Slack, 1991b). This then raises the possibility that when the vegetal explants were taken out of the embryo and grown in isolation, some of the cells on the outside of the explant were induced by a lower concentration of the inducing signal pushing it towards the mesodermal fate. In other words even if no fated mesoderm were present in the vegetal explants, some mesoderm become specified due to this embryonic regulation. It should be noted that this model of mesoderm induction is not consistent with the "ratchet" principle of posterior dominance discussed previously which also applies to the vegetal signals in early embryo. However the mechanisms of development *in vivo* is not always simple and may involve mechanisms that might initially seem incompatible.

To avoid including mesodermal cells in the endodermal explants, Horb and Slack (2001) in their study used explants made from stage 20-23 embryo. At this stage of development mesoderm and endoderm have formed distinct layers and can easily be separated. Indeed endoderm-only explants from stage 20-23 embryo do not show expression of any of the mesodermal markers (*FoxF1*, *XNkx-2.5*, *XTbx5* and *xFOG*). Horb and Slack (2001) then demonstrated that these endoderm-only explants grow to form unspecified endodermal cell masses that do not express the specification markers *Xlhbox8* or *Xcad2* but do express the pan endodermal marker *Edd*. Furthermore when the endoderm was cultured with the mesoderm still attached, the explants went on to express both the *Xlhbox8* and *Xcad2* specification markers (Horb and Slack, 2001). This then provides evidence that the specification of the endoderm in *Xenopus* takes place later than originally thought and that the mesoderm plays an important role in it.

I.6.3. Nature of mesodermal signals

The results outlined above showed that mesoderm plays a crucial role in the specification of the endoderm. However it was not yet clear whether the mesoderm is simply reinforcing a prepattern in the endoderm (permissive) or whether it is actively instructing the specification of the endoderm (instructive). To address this mesendodermal recombinations was carried out. Anterior endoderm was recombined with posterior mesoderm explants and vice versa, if the endoderm maintains its original fate then the mesoderm plays a permissive role, and if the endoderm takes the positional identity of its newly recombined mesoderm then the mesoderm would be sending out instructive signals. Horb and Slack showed that the recombinations resulted in the endoderm being respecified. In other words when recombined with an anterior mesoderm the endoderm would go on to express *Xlhbox8* and inhibit *Xcad2*, on the other hand when recombined with posterior mesoderm the endoderm would show inhibition of *Xlhbox8* and induction of *Xcad2* expression. (Horb and Slack, 2001).

I.6.4. Timing of regional specification

Studies in *Xenopus* have suggested that the regional specification of the endoderm becomes stable somewhere around stage 25. This can be seen as before stage 25 the expression of *Xlhbox8* and *Xcad2* is diminished when the mesoderm is removed. It has been suggested that this labile expression of the specification markers before stage 25 is due in part to the relative movement of the germ layers that takes place during neurulation. Comparison of the fate map of endoderm and mesoderm of *Xenopus* has shown that only the future epithelium and smooth muscle layer in the middle of the gut overlay each other in early embryo. Whilst those which are at the proximal end (e.g. oesophagus and stomach) and distal end (e.g. large instestine) do not align. (Chalmers and Slack, 2000). This is similar to the finding with the chick fate map where the presumptive endoderm are located in a more anterior position than its corresponding mesoderm and only becomes aligned as the gut tube closes (see section 1.5.1, p14) (Matsushita, 1995; Matsushita, 1996).

This hypothesis for the late stabilisation of endoderm patterning is supported by several other observations of late specification in *Xenopus*. For example, transgenic experiments in our lab have shown that an elastase promoter (marker of liver development) becomes activated about stage 31 (Beck and Slack, 1999). Also even though *Xlhbox8* mRNA is expressed as early as stage 10.5 in *Xenopus*, its protein is only visible late at stage 33 (Horb and Slack, 2001; Wright et al., 1988; Wright et al., 1989). The *Xenopus* embryo has also been shown to develop abnormalities in the gut when treated with retinoic acid between stage 25-35. Indicating that the endoderm is still being specified at this stage (Zeynali and Dixon, 1998). Endoderm isolated prior to stage 28 when grafted to a new location takes a new identity according to their new position. While grafts made with endoderm older than stage 28 retain their original identity (Zeynali et al., 2000). Finally, expression of high levels of *Xlhbox8*, *insulin, LFABP*, and *IFABP* mRNA do not begin until stages 30-35 (Horb and Slack, 2001).

I.6.5. Studying the mesodermal signals in *Xenopus*

The relatively recent discovery of the role of mesoderm in endoderm specification in *Xenopus* (Horb and Slack, 2001) has brought attention back to the study of gut development. Thus far only the existence of the mesodermal signals have been established in *Xenopus*. This study then proposed to follow up on the work done by Horb and Slack (2001) to try and identify the instructive signals being sent by the mesoderm.

Experimental systems using embryo tissue isolation and culture have proven valuable in previously determining the mesodermal signals in chick (Kumar et al., 2003) and mouse (Wells and Melton, 2000). Such a system in *Xenopus* has already been established. In his study Horb showed that endoderm explants could be made from embryos between stage 20 and 23 and be kept in culture long enough to assay for specification either by PCR or by *in situ* (Horb and Slack, 2001).

Xenopus also have several advantages compared to studies done in chick and mouse. From a practical point, the generation of endoderm explants from *Xenopus* should be relatively easy as the embryos are easy to generate, develop externally and are large enough to allow easy manipulation, thus making it ideal for conducting such a growth factor screen.

In avian systems it is not possible to prevent early contact between presumptive gut mesoderm and endoderm in generating the necessary explants (Rawdon, 2001). Similarly in mouse, the isolated endoderm generated from E7.5 embryo showed low level expression of *Tbra*, a mesodermal marker (Wells and Melton, 2000). However in *Xenopus* this should not be a problem. As demonstrated previously, endoderm explants from stage 20-23 embryo can be made to be free of any contaminating mesoderm (Horb and Slack, 2001). This is important as mesoderm contamination would lead to a complication in interpreting any results coming out of the screen.

I.7. *Hox* genes

From the above discussions on endoderm specification in mouse, chick and *Xenopus* we see that the mesoderm is essential for the complete specifcation of the endoderm (Dessimoz et al., 2006; Horb and Slack, 2001; Kumar et al., 2003; Wells and Melton, 2000). It is thought that the mesoderm sends out different signals to specify different parts of the endoderm depending on its position along A-P axis. In order to do this the mesoderm must be able to tell its own positional identity along the A-P axis. It has been suggested that the *Hox* genes might be involved in providing this positional identity in the mesoderm.

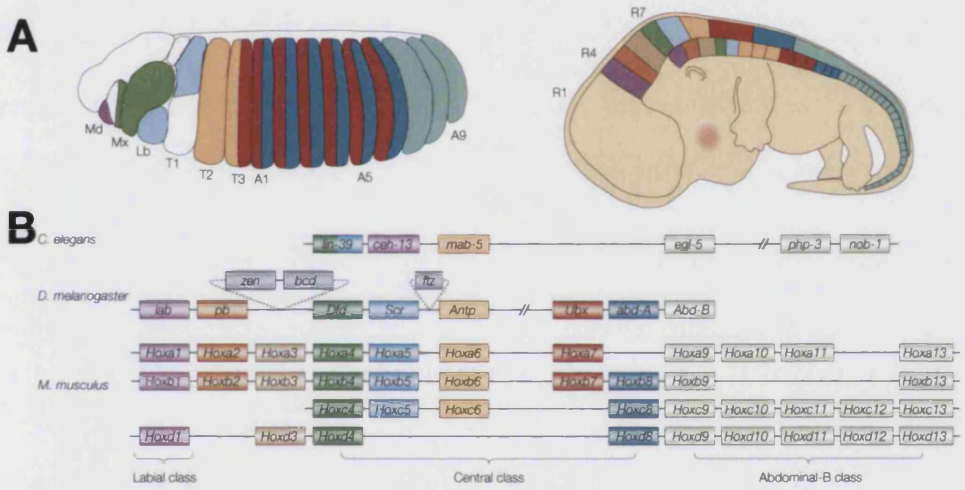

Figure 1.7 Hox Genes clusters and its expression pattern.
Taken from (Pearson et al., 2005) [3]. A) The panel on the left shows a stage 13 *Drosophila melanogaster* embryo that has been coloured in the schematic to indicate the approximate domains of transcription expression for all *Hox* genes. The panel on the right shows a mouse (*Mus musculus*) embryo, at embryonic day 12.5, with approximate *Hox* expression domains depicted on the head–tail axis of the embryo. In both diagrams the colours that denote the expression patterns of the *Hox* transcripts are colour-coded to the genes in the *Hox* cluster diagrams shown in B. B) A schematic of the Hox gene clusters (not to scale) in the genomes of *Caenorhabditis elegans*, *D. melanogaster* and *M. musculus*. Gene colours differentiate *Hox* families, orthologous gens are labeled in the same colour.

[3] Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Genetics, (Pearson et al., 2005), copyright (2005).

The *Hox* genes are a subset of the *homeobox* genes which encode transciption factors that contain a 60 amino-acid domain (the homeodomain). Members of the *Hox* genes are physically linked on a chromosome in clusters, consisting of 8 homeotic genes in the split Antennapedia-Ultrabithorax complex of *Drosophila melanogaster* and 39 *Hox* genes in 4 complexes in mammals.

An interesting property of the *Hox* genes is that the gene order in the cluster mimics the order of expression of genes and their function along the A-P body axis: genes found at the 5' end of the cluster are expressed in, and pattern, the posterior part of the body, whereas genes at the 3' end pattern the anterior end of the body (Fig. 1.7) (McGinnis and Krumlauf, 1992). This unique phenomenon of the *Hox* genes is known as collinearity.

However recent evidence indicates that this conventional view of *Hox* gene arrangement may not be true for all animals. Studies done on the increasing number of animal genome sequences have shown that *Hox* gene clusters are actually either fragmented, reduced, or expanded in many animals instead of being arranged in a collinear fashion (Lemons and McGinnis, 2006).

Another interesting feature of *Hox* genes is that the more posterior *Hox* genes seem to be dominant over the anterior *Hox* genes. In the canonical view, *Hox* genes are expressed from a different anterior boundary towards the posterior end of the animal. Ectopic expression of *Hox* genes anterior to their normal anterior boundary results in that segment taking on the more posterior fate of the *Hox* gene. However if an anterior *Hox* gene is expressed in a position more posterior than its anterior boundary, no respecification takes place. This phenomenon is known as "posterior dominance" (Manak and Scott, 1994). This principle often times, also holds true with non-canonical *Hox* genes where the gene is not expressed until the posterior end. Due to this, overexpression of *Hox* genes usually result in posteriorisation of the affected tissues whilst knockdown studies result in an anteriorisation of the affected tissues.

Hox genes have been known to be crucial in patterning the animal body along the A-P axes. Indeed mutations in the *Hox* genes have been shown to result in morphological defects that are restricted to discrete segmental zones along (A-P) axes in a wide variety of animals, ranging from nematodes to mice (Pearson et al., 2005). Several studies have shown that *Hox* genes provide positional information along the A-P axis in the development of mesoderm and ectoderm. In the mesoderm, the shape of a vertebra is controlled by *Hox* code, and in the hindbrain, appropriate neuronal differentiation is *Hox*-dependent (Krumlauf, 1994; McGinnis and Krumlauf, 1992).

I.8. *Hox* genes and the developing gut

I.8.1. *Hox* genes and the endoderm

The number of *Hox* genes expressed in the endoderm is not as numerous as those found expressed in the mesoderm. In *Drosophila* only *labial* is found to be expressed in the endoderm (Bienz, 1997). The case is similar with chick embryos where most are expressed in the mesoderm whilst only a subset is expressed in the endoderm at levels detectable by *in situ* hybridisation (Fig 1.8A) (Grapin-Botton, 2005). In *Xenopus* only one of the *abdominal-B type Hox* gene, x*HoxA13*, is thought to be expressed in the endoderm (Lombardo and Slack, 2001)

The expression boundaries of the *Hox* genes in the endoderm do not always correlate with boundaries between organs (Grapin-Botton, 2005). However *Hox* boundaries can be seen at the level of sphincters (pyloric, ileocaecal, anal) (Roberts, 2000). Inactivation of the endodermal *Hox* genes: *Hoxa-3* (Manley and Capecchi, 1995; Manley and Capecchi, 1998), *Hoxa-4* (Tennyson et al., 1993; Tennyson et al., 1998), *Hoxa-5* (Manley and Capecchi, 1995; Manley and Capecchi, 1998), *Hoxc-4* (Boulet and Capecchi, 1996) and *Hoxa-13* and *Hox d-13* (Warot et al., 1997) have been shown to lead to malformations of the digestive tract. But in all these cases no homeotic transformations of the gut structure was reported, possibly due to redundancy in the *Hox* signalling (Grapin-Botton, 2005).

It is worth noting that in the endoderm, another homeobox gene-complex, the *ParaHox* cluster was also expressed. In Amphioxus this cluster, which contain homologues of the genomic screened homeobox (*Gsh1*) gene, *Pdx1* and *Cdx2*, have been suggested to have a possible role in the A-P patterning of endoderm (Brooke et al., 1998). This cluster is not exclusively expressed in endoderm although most of its effect seems to involve endoderm development. *Pdx1* and *Cdx2* have been shown to be responsible for the development of pancreas and intestine respectively (Grapin-Botton and Melton, 2000; Kim et al., 1997; van den Akker et al., 2002). These three members of the *Parahox* cluster are expressed orderly along the A-P axis, *Gsh1* being the most anterior and *Cdx2* the most posterior(Grapin-Botton, 2005).

There is a suggestion that these genes might act as repressors of some *Hox* genes since the posterior expression boundaries of *Hoxa-3* and *Hoxb-4* seem to correspond quite well with the anterior limits of *Pdx1* and *Cdx2* expression (Grapin-Botton, 2005). Gene inactivation of *Cdx2* results in homeotic transformations. *Cdx2* heterozygous mutant mice exhibit induction of stomach tissue in the midgut and hindgut (Beck et al., 1999). *Pdx1* inactivation results in absence of the pancreas and defects in the most anterior duodenum.

I.8.2. *Hox* genes and the mesoderm

In *Drosophila* most of the *Hox* genes are regionally expressed in the mesoderm, with the exception of *labial* which is expressed in the endoderm, and have been shown to control the regional secretion of *Decapentaplegic (Dpp)*, a member of the *TGF-ß* family, and *wingless (Wg)*, a *Wnt* molecule. Both these molecules signal to the adjacent endoderm and facilitate its patterning (Bienz, 1997). In fact in the developing midgut of the *Drosophila* endoderm they have been shown to actually influence the expression domain of the only *Hox* gene in the endoderm, *labial* (Bienz, 1997).

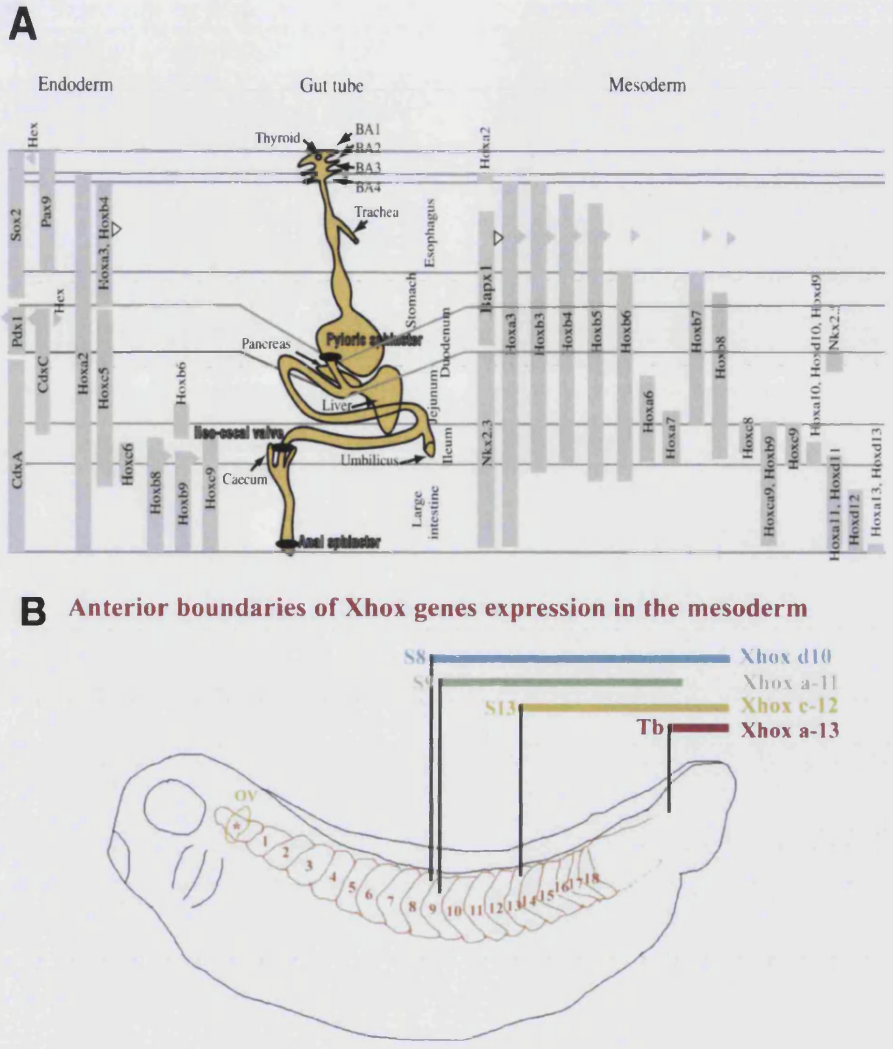

Figure 1.8 Regional expression of hox genes in chick and *Xenopus* mesoderm. A.) Chick - taken from (Grapin-Botton, 2005)[4]. Shows expression boundaries of *Hox* genes in chick mesoderm compiled from results in chick embryonic day 4 (Grapin-Botton and Melton, 2000; Sakiyama et al., 2000; Sakiyama et al., 2001; Yokouchi et al., 1995). B.) *Xenopus* - taken from (Lombardo and Slack, 2001)[5]. Shows anterior boundaries of several abdominal-B type *Hox* genes on stage 32 embryo.

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[4] Reprinted from Dev Biol, 49, Grapin-Botton, Antero-posterior patterning of the vertebrate digestive tract: 40 years after Nicole Le Douarin's PhD thesis, 335-47, Copyright (2005), with permission from Elsevier

[5] Reprinted from Mech Dev, 106, Lombardo and Slack, *Abdominal B-type Hox* gene expression in *Xenopus laevis*, 191-5., Copyright (2001), with permission from Elsevier

Several *in situ* studies in chick have found the *Hox* genes to be expressed in a nested, overlapping pattern in the developing gut mesoderm (Roberts et al., 1995; Sakiyama et al., 2000; Sakiyama et al., 2001; Yokouchi et al., 1995). Furthermore, unlike with *Hox* genes expressed in the endoderm, the boundaries of expression pattern of some of these mesodermal *Hox* genes match the morphological borders of the different gut regions (Fig. 1.7A) (Grapin-Botton, 2005). This is especially true for the 5' members of the *Hoxa* and *Hoxd* clusters (paralogues 9-13) (Roberts et al., 1995). The expression pattern of these *Hox* genes have been shown to match the morphological borders of the different gut regions of the posterior midgut and hindgut. This then strongly supports the notion that they play a role in facilitating endoderm specification by the mesoderm.

Indeed the misexpression of *Hoxd-13* in chick mesoderm has been shown to be able to induce a hindgut fate on midgut tissue (Roberts et al., 1998). This is further supported with murine transgenic experiments where inactivation or misexpression of the *Hox* genes result in abnormal gut development (Pollock et al., 1992; Wolgemuth et al., 1989). In particular, loss of expression of *Hoxa-13* and *Hoxd-13* have resulted in the alteration of muscle layers of the sphincter, which is consistent with a partial anterior transformation of this region (Kondo et al., 1996; Warot et al., 1997)

In *Xenopus* a similar regional expression pattern is also observed with a number of abdominal type B *Hox* genes (Fig. 1.8B) (Lombardo and Slack, 2001). Eventhough these expression patterns have not been matched with morphological boundary of the gut in *Xenopus*, it certainly raises the possibility that they might provide the mesoderm with positional information along the A-P axis this allowing it to specify the mesoderm.

I.9. Aims

I.9.1. Identifying the mesodermal signals in *Xenopus*

The relatively recent discovery of the role of mesoderm in endoderm specification in *Xenopus* (Horb and Slack, 2001) challenges the previous

cell-autonomous model of endoderm specification. In this new mesoderm dependent model of endoderm specification, only the existence of the mesodermal signals have been established. This study then was designed to follow up on the work done by Horb and Slack (2001) to try and identify the instructive signals being sent by the mesoderm.

This study aimed to address this by adapting the explant culture system from stage 20-23 embryo and developing a screen through which a number of commercially available growth factors would be tested. Through the screen growth factors which elicit an effect on endoderm specification would be identified for further study. Any growth factor that plays a role in endoderm specification would be detected by a shift or change in the expression of either *Xlhbox8* or *Xcad2,* anterior and posterior markers of development respectively in *Xenopus*. We expected to be able to isolate several growth factors through this screen to characterise and study further.

Advantages of using a *Xenopus* system to study mesodermal signals in endoderm development have been discussed in Section 1.6.5 (p27). The relatively easy generation of endodermal explants and assurance that it would be free of mesoderm should allow us to do a more conclusive analysis of the mesodermal endodermal interaction in regional specification compared to other studies done in mouse and chick.

I.9.2. Overexpression of *Hox* Genes

Most of the studies done regarding endoderm development in vertebrate system have concentrated on the mesoderm and endoderm relationship without addressing the underlying factors responsible for the A-P patterning. *Hox* genes have been shown to be expressed in the mesoderm around the developing gut in nested overlapping regions in *Xenopus* and chick around the time of regional specification. Since they have been shown to provide positional information along the A-P axis in the development of mesoderm and ectoderm there is a possibility that they also

are responsible for initiating the A-P patterning activities of the mesoderm on the endoderm.

This study aimed to explore this particular possibility by using RNA injection to overexpress the *Hox* genes to explore its possible function in this aspect of development. It was hoped that the misexpression of *Hox* genes would lead to a change in the positional identity of the mesoderm which in turn would lead to the misspecification of the endoderm. Detection of this change would be checked using similar PCR and *in situ* techniques as before for *Xlhbox8* and *Xcad2*, anterior and posterior markers of development respectively.

The study would be focused on the more posteriorly expressed of the *Hox* genes. This is because so far the studies in both chick and mouse have indicated a posterior based specification of the endoderm (Dessimoz et al., 2006; Kumar et al., 2003; Wells and Melton, 2000). Also posterior *Hox* genes are known to be more dominant than their anterior ones (Manak and Scott, 1994). These two observations then would suggest that posteriorising the endoderm would be more feasible than anteriorising it through *Hox* overexpression.

Lastly, we must note that during the course of work on this study, there was significant progress made in the study of endoderm specification in chick and mouse systems, which have been referred to and discussed above. It should be taken into account when looking at the experimental chapters of this thesis that most of these results were unavailable when this work started in 2003.

II. Materials and Methods

II.1. Xenopus husbandry

Adult male and female Xenopus were maintained on a diet of pellets (Blades Biological), braised steak and maggots. They were kept in a circulating water aquarium at 21°C and exposed to a daily 12 hour light and 12 hour dark lighting cycle. To procure eggs the female *Xenopus* was primed with an injection of 40 units of Pregnant Mare Serum Gonadotrophin (PMSG, Calbiochem) into the dorsal lymph sac from anything between 1 day to a week before the eggs were needed. This helps increase the quality and quantity of eggs obtained for fertilisation. To induce egg laying itself the females were injected with 300 - 600 units of Human Chroionic Gonadotrophin (Chorulon, Intervet) the evening before the eggs were required.

II.2. *Xenopus* embryos artificial fertilisation

In order to obtain the sperm necessary for artificial fertilisation a male *Xenopus* was killed using a Home Office approved schedule one procedure. This usually involved treating the male with an overdose of anasthaetic (10% benzocaine in ethanol) for at least 30 minutes. After the male had died the abdomen was cut open and the testes were removed. The testis, once removed was kept moist by putting it on top of a filter paper soaked in NAM/2 medium (See Table 2.1 for composition of NAM solutions). For longer storage (up to 1 week) the testes were kept in NAM/2 in 4°C. This kept the sperm in the testis viable for future fertilisation.

Before it could be used in artificial fertilisation the testis needed to be 'teased'. This was done by making several slices in the testis and subsequently pinching it several times using forceps (No.5 Jewellers forceps, Sigma) or scissors. This helped release the sperm necessary for artificial fertilisation. At this point *Xenopus* eggs were collected from the

injected females by the application of gentle pressure on their abdomen towards the cloaca.

To fertilise the collected eggs, 'teased' testes was passed over them. This was done gently as the eggs can become activated by rough handling. The eggs were left untouched for about 5 minutes and then submerged in milliQ water. The embryos were then left for another 20 minutes, at this point the eggs should have rotated with the animal caps pointing up. Once rotated the eggs were dejellied using 2% Cysteine HCl at pH 7.8-8.0, wash 2 or 3 times with Mili-Q water and was then transfered to grow onto a Petri dish with NAM/10 solution.

Table 2.1 NAM solutions

	NAM	**NAM/2**	**NAM/10**
NaCl	110 mM	55 mM	11 mM
KCl	2 mM	1 mM	0.2 mM
$Ca(NO_3)_2.4H_2O$	1 mM	0.5 mM	0.1 mM
$MgSO_4.7H_2O$	1 mM	0.5 mM	0.1 mM
Sodium EDTA	0.1 mM	0.05 mM	0.01 mM
Sodium Hepes	5 mM	5 mM	5 mM
Sodium bicarbonate	1 mM	1 mM	-------
Gentamicin sulphate	2.5 µg/ml	2.5 µg/ml	2.5 µg/ml

Note: Gentamicin is a general antibiotic used to prevent infections.

II.3. RT-PCR

II.3.1. Reverse Transcription

RNA was prepared from the collected tissue samples by homogenising them in Trizol (Invitrogen). The quality and relative quantity of the isolated RNA was checked by running them on a 2% gel. The gel should show the two bright ribosomal band and no smear coming from genomic DNA contamination. The concentration of the extracted RNA was checked using a spectophotometer.

Before performing reverse transcription, the RNA sample was first treated with RNase free DNaseI (invitrogen) to help further reduce possible genomic DNA contamination before they were reverse transcribed using the Superscript III reverse transcription kit from Invitrogen. Approximately 1-2 µg of RNA was used for each reverse transcription reaction.

Table 2.2 PCR Primers

PCR Target	Sequence	Tm	Cycles
EF1α Forward	5' AGATTGGTGCTGGATATG 3'	58°C	20
EF1α Reverse	5' ACTGCCTTGATGACTCCTA 3'		
Xcad2 Forward	5' CCACCAACGGTAAGACAA 3'	58°C	25
Xcad2 Reverse	5' GGAGATACCAAGTTGCTG 3'		
Xlhbox8 Forward	5' TGCCAACTTCATCCCAGCCC 3'	58°C	25
Xlhbox8 Reverse	5' GGCAGATGAAGAGGGCTC 3'		
*FoxF1*Forward	5' AACCCTCTGTCCTCCAGCCT 3'	58°C	25
*FoxF1*Reverse	5' GGTTAGTGGAATGACTAACTTC 3'		
xFOG Forward	5' TATGCCCAGAAGTTACAGGAA 3'	58°C	25
xFOG Reverse	5' CACCTCCTTTTTGTGCCAGTG 3'		

II.3.2. Polymerase Chain Reaction (PCR)

PCR was performed using a Red PCR mastermix (ABGene) or using separate components of Taq, dNTPs and buffer (Invitrogen). Table 2.2 on the previous page lists the Primers used in this study along with annealing temperatures and cycle numbers for each primer pair.

Before cDNAs from the reverse transciption reaction were used, they were first treated with RNAseH (Invitrogen) to destroy the template mRNA. This reduced any possible false positives in the PCR reaction. Approximately 1-1.5 µl of the reverse transcription reaction was used for each PCR reaction. Resulting PCR products are run on 1.2-1.5% Agarose gel with Ethidium Bromide. At least half of the PCR reaction was loaded on the gel.

II.4. *In situ* Hybridisation

II.4.1. Generating DIG labelled probes

To generate the appropriate DIG labelled RNA probe for wholemount *in situ* hybridisation the following labelling reaction mix is prepared. At room temperature in a sterile RNase free eppendorf:

DEPC treated Mili-Q water	to 50 µl
5x transcription buffer (Promega)	10 µl
100mM DTT (Promega)	5 µl
10x DIG-NTP mix (Roche)	2.5 µl
Linear DNA template	2.5 µg
RNAout (Invitrogen)	2 µl
RNA polymerase (SP6, T3 or T7 from Promega)	2 µl

DEPC water is prepared by adding 0.1% (v/v) DEPC to Mili-Q water allowing the water to stand overnight and subsequently autoclaved. The RNA polymerase was chosen based on the plasmid that the probe is generated from (see below). Note that the DIG-NTP mix only needs to be added at half strength to save on resources.

The above prepared mix was then allowed to incubate at 37°C for about 4 hours. Additional RNA polymerase (2µl) was added halfway through this incubation to help increase yield. After this incubation period the DNA template was removed by adding 2.5 µl RNase free DNaseI (Promega) for 20 minutes at 37°C.

The resulting RNA probe was then purified by passing them through a G50 sephadex column (Amersham Bioscience). This column allows the RNA probe to go through but not the free dNTPs. The column was prepared by vortexing for 30 seconds, and then with the cap half open centrifuged at 1000g (3000 rpm on tabletop centrifuge) for 1 minute to remove the storage solution. The RNA sample was then applied to this prepared column and then run again at 1000g for 2 minutes. The collected solution was then run on a 2% gel to check for quality. A good synthesis should yield a smeared band on the gel. Concentration of the RNA product was checked with spectophotometer. The RNA probe can be stored in either -20°C or -80°C.

In this study the following plasmid was used to generate DIG probes: *Endodermin* (Linearise with EcoRI/ transcribe with T7), *Xlhbox8* (EcoRI/T7), *Xcad2*-pCS2+ (NotI,T7). After complete linearization with the appropriate enzyme the DNA was isolated using Gel-clean up kit from Promega.

With constructs that are in pCS2+ it is preferable to prepare the template for RNA transcription via PCR. This is because pCS2+ has a mutated T7 promoter that prevents a good RNA transcript from being made from this promoter. To overcome this PCR was performed on the template on pCS2+ with an SP6 primer and a T7 primer with a corrected promoter sequence. Then 5 µl of the resulting PCR reaction were used as follows:

SP6 primer 5' CTATAGTGTCACCTAAATAGCTTTGGCG 3'

Corrected T7 primer 5' GTAATACGACTCACTATAGGTC 3'

The PCR reaction was performed with a melting temperature of 50°C for 25 cycles. The low temperature should allow for the mismatch at the T7

promoter to hybridise with the template and ensure a good PCR amplification.

II.4.2. Specimen preparations

Specimens meant for *in situ* hybridisation were fixed for 1 hour at room temprature using MEMFA (0.1M MOPS (pH 7.2), 2mM EGTA, 1mM MgSO4 and 10% (v/v) formaldehyde pH 7.4). After fixation the specimens were then washed for 5 minutes in 100% EtOH and then placed in fresh 100% EtOH and stored at -20°C. The specimens can be stored in this manner for up to 3 months.

II.4.3. *In situ* Hybridisation

The *in situ* hybridisation was performed as described in (Harland, 1991) over three days with minor modifications that will be described below. The first day involves the hybridisation of the DIG RNA probe. The first day protocol was carried out using 5ml glass vials placed on a nutator (Fisher) for agitation. The specimens that have been fixed and kept in 100% EtOH are first rehydrated through a series of rehydration washes: 75% EtOH in PBSAT (PBS + 0.1% Tween) for 5 minutes, 50% EtOH in PBSAT another 5 minutes and three 5 minute washes in PBSAT. Once rehydrated the specimens were then treated with Proteinase K (10 µg/ml) for 10-20 minutes, depending on the specimen size, to help with permeability. Care is taken not to agitate the specimen too harshly when treating with Proteinase K as it will result in excessive damage to the specimens. After Proteinase K treatment the samples are then washed twice with triethanolamine (0.1 M, pH 7.8) for 5 minutes each on the nutator. At the end of the second wash 12.5 µl of acetic anhydride was added to each of them. For this step to avoid damage they were not agitated on the nutator, but were instead swirled gently and incubated for 5 minutes. Another 12.5 µl of acetic anhydride were added and samples incubated for another 5 minutes. The acetylation with acetic anhydride reduces non-specific binding to NH_2 groups. The specimens were then washed twice for 5 minutes each in PBSAT and then refixed with 10% (v/v) formaldehyde in PBSAT for 20

minutes. After fixation they are washed again with PBSAT 5 times for 5 minutes each to remove traces of formaldehyde. At the end of the fifth wash the vials were filled with 1 ml of PBSAT + 250 µl of hybridisation buffer. Hybridisation buffer was made by combining the following:

	Final conc	**add**
Formamide	50 %	25 ml
20x SSC	5x	12.5 ml
50 mg/ml yeast RNA	1 mg/ml	1 ml
100 mg/ml heparin	100 µg/ml	50 µl
50x Denhardt's	1x	1 ml
20% tween 20	0.1%	250 µl
20% CHAPS	0.1%	250 µl
0 .5 M EDTA pH 8	10 mM	1 ml

Next the specimens were transferred into new vials containing 1-2 ml of hybridisation buffer, then incubated at 60°C for 10 minutes. After this the hybridisation buffer was replaced and specimens incubated for a further two hours at 60°C. This pre-hybridisation step is important as it blocks non-specific sites in the specimens. The DIG-RNA probes were prepared at 1 µg/ml concentration in hybridisation buffer and were preheated to 60°C before adding it to the specimens at the end of the pre-hybridisation step. Once the DIG-RNA probes have been added to the vials, they were incubated at 60°C overnight.

On day two of the *in situ* hybridisation, DIG-RNA probe that had been left to hybridise overnight was removed and was replaced with fresh hybridisation buffer and then left to incubate for 15 minutes at 60°C. Solution exchanges were performed in the 60°C water bath to prevent temperature loss and thus maintain the stringency of the hybridisation. The DIG-RNA probe that was removed can be stored at -80°C for up to 2-3 further hybridisations. After 15 minutes the specimens were put through a series of washes. These comprise of three washes with 2xSSC + 0.1% Tween 20 for 20 minutes each at 60°C and a further two washes with

0.2xSSC + 0.1% Tween 20 for 30 minutes each at 60°C. All of the solution exchanges above were performed in the 60°C water bath to prevent temperature loss. Next the specimens are removed from the 60°C bath and brought to room temperature where they underwent two washes in Maleic acid buffer (MAB, 100 mM Maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.8) for 15 minutes each. This was followed by blocking step for 30 minutes in MAB + 0.1% Tween 20+ 2% Boehringer Mannheim Blocking reagent (Roche) for 30 minutes and another 2 hours in MAB + 0.1% Tween 20+ 2% Boehringer Mannheim Blocking reagent + 20% heat treated sheep serum. After the blocking step was finished the DIG antibody was added at 1:2000 dilution in MAB + 0.1% Tween 20+ 2% Boehringer Mannheim Blocking reagent + 20% heat treated sheep serum. The antibody was incubated overnight at 4°C.

Day three consists mainly of washes, the antibody was removed from the specimens and the specimens were washed with MAB + 0.1% Tween 20 at room temperature three times for 15 minutes each and another six times at 30 minutes each. At the end of the washes the solutions were replaced with alkaline phosphatase buffer (100 mM Tris Cl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20) for 3 and 10 minutes washes. The alkaline phosphatase buffer is replaced with BM purple solution (Roche) and left undisturbed until colour had developed. The length of this incubation varied in some cases from 1 hour to overnight depending on the abundance of the target mRNA. If overnight development was required the specimens were placed in the dark or in 4°C. 5 mM Levamisole was added to the alkaline phosphatase buffer and BM purple to further inhibit endogenous alkaline phosphatase activity and reduce background if necessary. Levamisole acts by inhibiting the non-intestinal isoform of alkaline phosphatase, which makes up most of the endogenous activity.

II.5. Microsurgery tools

Proper tools are necessary to ensure good microsurgery on Xenopus. These consist of 2 sharpened no.5 watchmakers' forceps (sigma) and 2

blunt watchmakers' forceps. The sharpened forceps were used to remove vitelline membranes from early embryos. They were made by sharpening stock no.5 watchmakers' forceps on a sharpening stone. It was important that the ends of the forceps meet exactly as otherwise it will not be able to grasp the vitelline membrane effectively (see Fig 2.1). Blunt forceps were used to move and orientate the embryos, the blunt edges help prevent damage to the embryo during this step

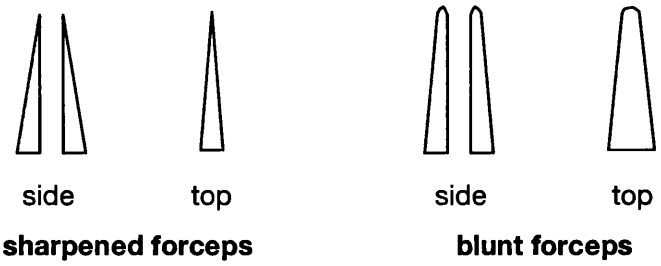


Figure 2.1 Sharp and blunt forceps
Diagrammatic representation of the forceps tips for both sharpened and blunt no.5 watchmakers' forceps.

To make incisions in the embryo a tungsten needle was used. Tungsten needles were made by sharpening tungsten wire through electrolysing in a 1M NaOH solution. A piece of plasticine was put at the end of the wire and as the tungsten was electrolysed the weight of the plasticine pulled on the wire until it breaks. This created a very sharp end to the wire which was suitable for making precise incisions on embryos. To make a tapered end to the needle the wire was moved in and out during electrolysis. The sharpened wire was then mounted in glass handles for easy handling. The sharp end of the needle was kept from contact with anything solid as this will result in a blunt needle.

Microsurgical manipulations of embryos were usually performed on 1.5% agar plates with NAM/2 solution (Table 2.1). The agar helps prevent damage to the sharp instruments and the high salt of NAM/2 facilitates the healing of the embryos after surgery.

II.6. Animal caps

Animal caps were made from stage 9-10 blastula embryos. First the vitelline membranes were removed from the stage 9-10 blastula embryos and then using either sharpened forceps or tungsten needles the animal cap was cut out from the embryo. Care was taken to ensure that only the thin animal caps are isolated, as the thick marginal tissue may contain mesoderm. The cut animal caps were directly transferred onto 2cm Petri dishes coated with 1.5% agar containing NAM/2 solution (Table 2.1) + 1%BSA. The animal caps were oriented with the dark outside cells touching the agar. The isolated animal caps are cultured in this way at 23°C for 3 days, changing the solution with fresh NAM/2 +1% BSA every 2 days.

II.7. Endoderm and Endoderm + Mesoderm explants

These are made from stage 20-23 embryos, the locations of the cuts to make the different explants are shown in figure 2.2 below. The microsurgery on these embryos is done using tungsten needles and performed in 1.5% agar plates in NAM/2 solution (Table 2.1). Cut 1 was made just behind the cement gland and marks the anterior end of the endoderm. Cut 2 was right before the proctodeum and marks the posterior end. Cut 3 was halfway between 1 and 2 in the middle of the embryo with cuts 4 and 5 made halfway between cuts 1 and 3 and 1 and 2, respectively. The explants were separated from the embryo by making a final cut along the edges of the shaded region shown in the diagram (Fig 2.2).

To make whole endoderm explants cuts 1 and 2 were made. To make half explants cuts 1, 2 and 3 were made. To further divide the endoderm into 4 regions from anterior to posterior cuts 1-5 were made. If endoderm free of mesoderm explants were to be made the cuts are done in NAM/2 in the presence of trypsin (1µg/µl, Sigma). This helps separate the two germ layers by degrading extracellular matrix material. Once separated the endoderm only explants were moved to NAM/2 solution containing trypsin inhibitor (2µg/µl, Sigma) and allowed to heal for approximately 0.5-1 hour. After they healed the explants were moved to a fresh NAM/2 +

1%BSA solution and cultured at 18°C. The culture solutions are changed with fresh NAM/2 + 1% BSA every 2 days. When making Endoderm + Mesoderm (endo+meso) explants, the cuts are performed in NAM/2 without trypsin. The explants were kept for half an hour to heal before moving them to NAM/2 +1% BSA solution for culture at 18°C. All the dishes used in explant culture are coated with 1.5% agar to prevent the endoderm sticking. The explants were cultured until the control embryos cultured alongside reached stage 41-42 (approx 4 days).

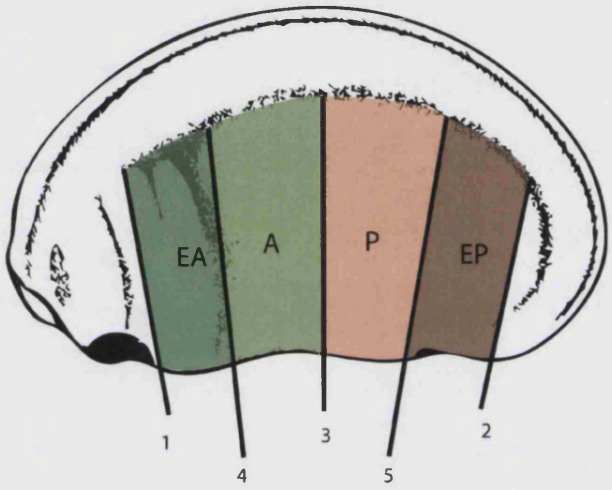


Figure 2.2 Stage 23 embryo showing locations of cuts.
Location of cuts to be made to make endodermal explants. If all cuts from 1 to 5 are made the endoderm is divided into four regions between it's A-P axis: Extreme Anterior (EA), Anterior (A), Posterior (P), Extreme Posterior (EP).

II.8. Isolating whole gut from stage 41-43 embryos

Embryos from between stage 41-43 were first immobilised by incubating with 1/5000 dilution of MS222. Once immobilised the epidermis surrounding the gut is removed using watchmakers' forceps to expose the gut. Then two cuts were made, one at the oesophagus and another at the cloaca to separate the gut from the embryo.

II.9. Growth Factor and Inhibitor Treatment

Treatment with growth factors and inhibitors on Endoderm or endo+meso explants and animal caps was done on 1.5% agar coated 2cm

petri dishes. The dishes were pre-incubated with NAM/2 + 1%BSA for approximately 15-20 minutes and this was then replaced with 2ml of NAM/2 + 1%BSA. The BSA acts as a carrier and prevent the plastic from absorbing too much of the growth factors. The growth factors or inhibitors were added to the solution at the appropriate concentration. The tissue to be treated was put on these plates and cultured at 18°C for Endoderm or endo+meso explants and at 23°C for animal caps.

For animal caps and Endoderm explants after 1 day of incubation the growth factor solution was replaced with fresh NAM/2 + 1%BSA. The solution was changed every 2 days until the experiment was finished. endo+meso explants were kept in growth factor solution in NAM/2 + 1% BSA throughout the culture period. New growth factor solution in NAM/2 + 1% BSA was put on the explants every 2 days.

An alternative way of applying the growth factor was by using heparin-acrylic beads (Sigma). Heparin-acrylic beads release growth factors slowly creating a graded concentration from the location of the beads, which mimics *in vivo* signals. Before implantation the heparin-acrylic beads were soaked with 1µg/ml of the protein for 2 hours at room temperature. This binds the protein onto the beads. After incubation the beads were washed three times with PBS. The beads were implanted through an incision made in the anterior of stage 20-23 embryo. Once the bead is inserted, the embryos were allowed to heal and cultured as for normal fertilised embryos.

For this study the growth factors ActivinA, BMP4, FGF4, FGF8, FGF10, EGF were obtained from R&D systems. Retinoic Acid (RA) and LiCl was obtained from Sigma. The inhibitors SU5402 and PD98059 were sourced from Calbiochem whilst sFRP2, Follistatin, noggin and cyclopamine were obtained from Sigma.

II.10. RNA / label injection

II.10.1. Preparing RNA for injection

Plasmids containing the constructs to be injected should be in the plasmid pCS2+ as RNA made from pCS2+ constructs have a polyA added that contributes to the stability of the mRNA after injection. RNAs for injections were made from plasmid constructs using the appropriate polymerase (Sp6 for constructs in pCS2+) mMessage machine RNA synthesis kit (Amersham). After synthesis the RNA was cleaned using G-50 sephadex column (Amersham) and diluted as necessary. Unused RNA can be stored at -20°C for approximately 1-2 months.

II.10.2. Injection procedure

RNAs or labels (BrdU/FDA) were injected using the Nanoject Injector (Drummond). Needles were prepared using a horizontal needlepuller. The tip of the pulled needle was clipped using forceps so that an opening is made no more than 10-20 µm diameter. The needle was then filled with mineral oil and loaded onto the injector. Once on the injector the needle was backfilled with solution containing the substance of interest. Injections were done in NAM/2 + 1% Ficoll (Sigma) solution in a 1.5% agar coated dish. Ficoll was added to the injection medium to help suppress the 'leakage' that would otherwise come out of the hole made by the needle. To target the injections the embryos were held with forceps and injected from the other side. The injected embryos were then allowed to heal in NAM/2 + 1% Ficoll for a minimum of 2-3 hours. Once healed the embryos were put in fresh NAM/10 solution and kept overnight at 18°C. Ficoll can cause failure of gastrulation and was removed to ensure normal development. The next day protruding tissues coming out of injection site were removed and the embryos were placed into fresh NAM/10 solution. The embryos were then treated as with normal artificially fertilised embryos.

II.11. Histology

II.11.1. Paraffin sections

Specimens were fixed in MEMFA (0.1M MOPS (pH 7.2), 2mM EGTA, 1mM $MgSO_4$ and 10% (v/v) formaldehyde pH 7.4) for an hour at room temperature and then placed in 70% ethanol before being processed for Wax embedding. They were processed by putting them through dehydration steps of 70% EtOH than 90% EtOH and twice in 100% EtOH. Then specimens were put into 2 incubations with histoclear and 3 incubations with molten Paraffin wax. For small specimens such as embryos from stage 35 or earlier each step was performed for 30 mins. For larger embryos (stage 38 or above) each step was performed for 1 hour each.

Once processed the specimens were embedded in wax and sectioned between 10-20 µm depending on the need. Thicker sections were sometimes needed to help visualise weak *in situ* signals. They were then put on polylysine superfrost slides (Fisher), dewaxed with histoclear. The specimens could now be counterstained with Ehrlich's haematoxylin solution (Sigma), dehydrated and mounted. For *in situ* specimens counterstaining was not necessary and the specimens are directly dewaxed with histoclear and mounted with Depex (National Diagnostics).

II.11.2. Cryosections

Specimens for cryosectioning were fixed in 4% PFA at room temperature for 1 hour (up to stage 35 embryo) or 2 hours (stage 40 or older embryo). They were then transferred to 15% sucrose in PBS for approximately 5 hours at 4°C until they sank and then 30% sucrose in PBS overnight at 4°C. The specimens were now embedded in with OCT (Sigma). They were flash frozen with dry ice and either sectioned directly or stored at -80°C for up to 1 month.

Cryosections were cut between 15-20 µm depending on need. Sectioned samples were dried at room temperature for approximately 2 hours. They were then washed in PBS before being mounted with Gelmount

(Biomeda). Gelmount is a water-miscible mountant that maintains signals in fluorescent samples and is necessary for GFP visualisation.

III. Growth Factor Expression pattern

III.1. Introduction

Growth factors are proteins which are present in animal tissues at very low concentrations and have high biological activity. They are typically found in specific places, usually close to the cells that produce them as a result of them being bound to cell surfaces or to extracellular materials (extracellular matrix). The growth, differentiation and survival of a cell depends largely on the composition of growth factor molecules in the microenvironment around it (Slack, 1991a). Due to the localised nature of growth factors, finding out where they are expressed can give valuable insight to their possible biological functions.

Spatial expression pattern have been previously used to help understand the roles of growth factors in endoderm specification. Experiments in chick and mouse embryos have shown that growth factors are able to mimic or replace mesodermal signals in endoderm specification (Dessimoz et al., 2006; Kumar et al., 2003; Wells and Melton, 2000). One of the growth factors tested, *FGF4*, was shown to pattern the endoderm in a posterior dominant fashion (Dessimoz et al., 2006; Wells and Melton, 2000). In the mouse *FGF4* has been previously shown to be expressed in the posterior of the embryo near the primitive streak (Niswander and Martin, 1992). In chick *FGF4* was also found to be expressed at high concentration near the posterior of the embryo after gastrulation, near the hindgut and midgut (Shamim and Mason, 1999). In *Xenopus* the closest homologue to *FGF4*, *eFGF* has a similar expression pattern during gastrulation (Isaacs et al., 1992b). In mouse and chick this posterior expression of *FGF4* was used in conjunction with the results from growth factor screens to draw up a

posterior morphogen model for the specification of the endoderm (Dessimoz et al., 2006; Wells and Melton, 2000).

The study done by Horb and Slack (2001) indicated that regional specification was not completed until after stage 25. Information regarding the expression pattern of the growth factors around stage 25 was incomplete. We aimed to address this by performing *in situs* on various different growth factors on stage 23 and 27 embryos. We had initially considered performing immunostaining alongside the *in situs* since it would give additional information as to where the growth factors maybe active. However this was difficult as antibodies for growth factor proteins in *Xenopus* that would work for whole mount immunostaining are not readily available.

III.2. Materials and Methods

In situ hybridisation probes for the growth factors were made by linearising the plasmid containing the gene with a restriction enzyme and synthesising the DIG probe with the appropriate RNA polymerase. Table 3.1 lists the restriction enzymes and polymerases used to generate the growth factor probes. *In situs* were then performed on stage 23 and 27 embryo.

Table 3.1 Enzymes and RNA polymerase for probe synthesis

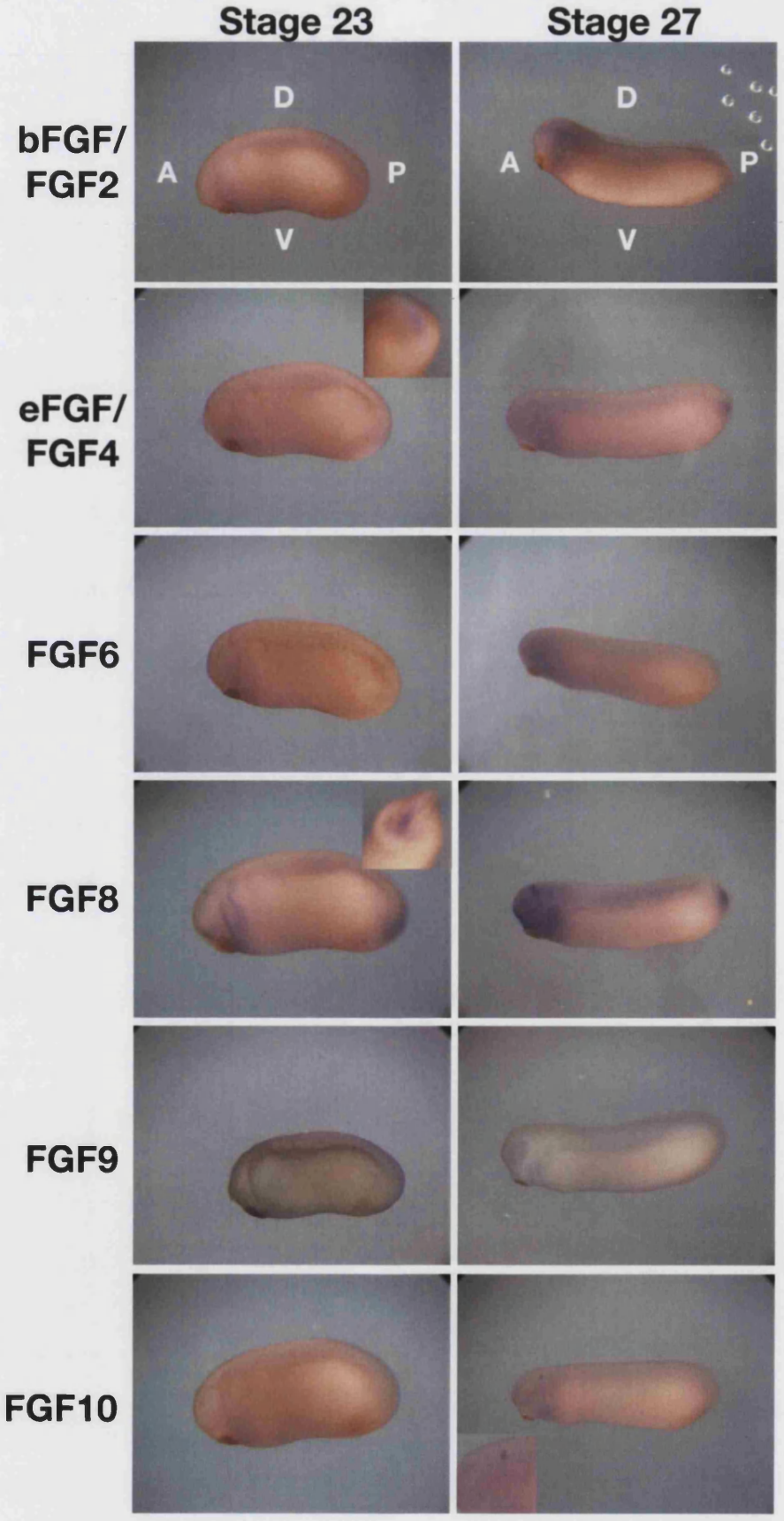
Target Gene	Restriction enzyme	RNA Polymerase
bFGF/FGF2	HindIII	T7
eFGF/FGF4	EcoRI	T3
FGF6	EcoRI	T3
FGF8	XbaI	T3
FGF9	SalI	T7
FGF10	EcoRI	T3
Wnt3A	ClaI	T7
Wnt5A	BamHI	SP6
Wnt7A	NotI	T7
Wnt8	AVAI	T7
BMP4	EcoRI	T3

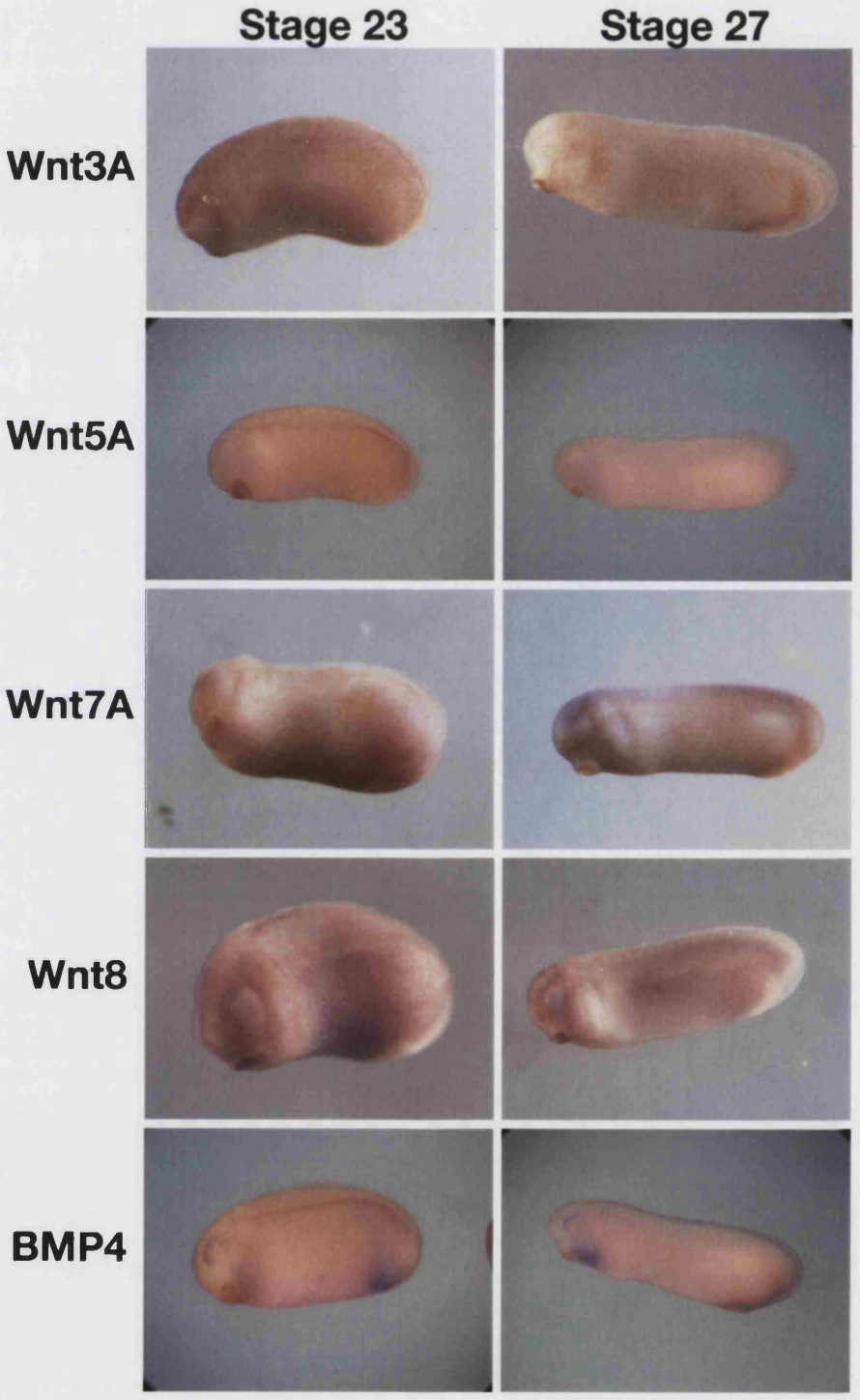
Once the *in situ* were fully developed the embryos were bleached with 2% H_2O_2 (Sigma) to allow better visualisation of the expression pattern.

III.3. Results

The *in situ* revealed that *eFGF/FGF4* and *FGF8* were expressed in posterior of the embryo. *eFGF/FGF4* and *FGF8* have been shown previously to be posteriorising factors which was consistent with the posterior expression pattern observed here (Christen and Slack, 1997; Isaacs et al., 1994; Pownall et al., 1996). *FGF8* also exhibited staining in the anterior of the endoderm around the branchial arches at stage 20 and 27 and the mid brain - hind brain boundary at stage 27. We do not think that this expression was relevant to the development of the endoderm. *FGF10* was seen to be expressed in the otic vesicle which was clear at stage 27 but not in the stage 23 embryo. No other domains were seen with the *FGF10 in situ*. *bFGF/FGF2*, *FGF6* and *FGF9* as well as the *Wnts* (*Wnt3A, Wnt5A, Wnt7A and Wnt8*) *in situ* did not show any relevant endodermal/mesodermal staining.

Figure 3.1 (next 2 pages) *In situ* on stage 23 and 27 embryo for probes *bFGF/FGF2, eFGF/FGF4, FGF6, FGF8, FGF9, FGF10, Wnt3, Wnt5A, Wnt7A, Wnt8.*
bFGF/FGF2 column shows the orientation of the embryos at stage 23 and 27: A=Anterior, P=Posterior, D=Dorsal, V=Ventral. The staining with *bFGF/FGF2* and *FGF6* did not show any relevant endodermal or mesodermal staining. *FGF9* shows a slightly blue overall staining with no apparent endodermal or mesodermal staining. eFGF/*FGF4* and *FGF8* were shown to be expressed in the posterior of the embryo at these stages. Inset in both *FGF4* and *FGF8* stage 23 panel shows a close up look at the posterior staining for these genes. *FGF8* also has a staining at the anterior of the embryo at the branchial arches at stage 20 and 27 and mid brain-hind brain boundary at stage 27. Whilst *FGF10* was expressed in the otic vesicle which can clearly be seen at stage 27 (see inset for a higher magnicifaction picture). *BMP4* is the most interesting with a possible endodermal expression pattern. The *Wnts* examined here do not seem to be expressed in either the endoderm or mesoderm around the gut.


Stage 23
Stage 27
bFGF/
FGF2
D
A
P
V
D
A
P
V
eFGF/
FGF4
FGF6
FGF8
FGF9
FGF10


Stage 23
Stage 27
Wnt3A
Wnt5A
Wnt7A
Wnt8
BMP4

In situ for *BMP4* showed that it was expressed near or around the endoderm in the anterior and posterior of the embryo. This may indicate a possible involvement in endoderm specification. From the whole mount *in situ* it was not possible to tell whether the *BMP4* expression was in the mesoderm or whether it was also expressed in the endoderm. To solve this we processed the whole mount *in situ* embryos, wax embedded and sectioned transversely.

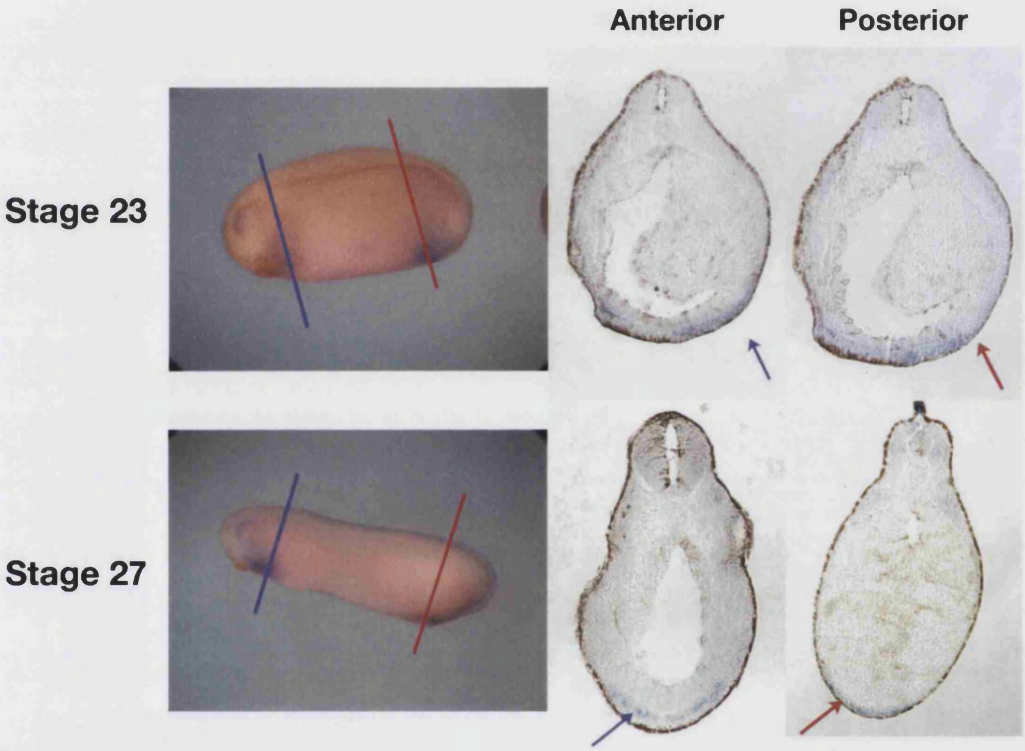


Figure 3.2 Sections of *BMP4* whole mount *in situ* for stage 20 and 27 embryo. Transverse sections are made of *BMP4* whole mount *in situ* at 10µm thickness. The blue line indicates position of the anterior sections whilst the red line shows the posistion of the posterior sections. Photographs of the sections are shown to the right. The arrows point the position of the *in situ* signal in the sections. Here we can see that the signal seems to be expressed in the mesoderm in both anterior and posterior sections.

Sections of the whole mount *in situ* for *BMP4* revealed that the staining was contained in a tight band near the outside of the embryo in both the anterior and posterior domains (Figure 3.2). This then showed that *BMP4* expression was in the mesoderm only

III.4. Discussion

eFGF/FGF4 and FGF8 were seen expressed towards the posterior of the embryos. This is consistent with the posteriorising roles of eFGF/*FGF4 and FGF8* previously seen in *Xenopus* (Christen and Slack, 1997; Isaacs et al., 1994). A posterior expression domain of *FGF4* have been seen in chick and mouse before (Niswander and Martin, 1992; Shamim and Mason, 1999). In both these organisms *FGF4* has been shown to be capable of posteriorising the endoderm (Dessimoz et al., 2006; Wells and Melton, 2000). This then raises the possibility that *FGF4* might posteriorise the endoderm as well in our growth factor screen (see Chapter IV)

At the moment there are no known functions of *FGF8* in endoderm specification. However *FGF8* has been implicated in the formation of somites in chick embryo. Regular spacing of somites is thought to involve a dynamic gradient of *FGF* signalling that controls the timing of maturation of cells in the presomitic mesoderm (PSM) (Delfini et al., 2005). *FGF8* has been shown to form a posterior morphogen gradient of mRNA along the A-P axis in the PSM of chick and mouse embryos through a mechanism of mRNA degradation (Dubrulle and Pourquie, 2004). In this mechanism *FGF8* transcription is restricted to the tail bud only. As the embryos elongate, descendants of the tail bud then inherits the *FGF8* mRNA. As the axis continues to elongate these cells are then pushed towards an increasingly anterior position. Taking into account RNA decay it can be seen how more anterior cells (older descendants) would have a lower level of FGF8 mRNA compared to more posterior cells (recent descendants), creating a posterior gradient of *FGF8* mRNA (Dubrulle and Pourquie, 2004).

The high levels of *FGF8* in the posterior of the embryo is thought to inhibit the differentiation of the PSM through the Erk/MAPK pathway. Because of the mechanism by which the gradient is formed, the highest level of *FGF8* is always in the tail bud. As the axis elongates the point at which the *FGF8* concentration stops inhibiting somitogenesis, the threshold, moves in a posterior direction. Thus as a result of this posterior movement

more and more posterior somites are formed, resulting in the regular spacing seen in the mature structure (Dubrulle and Pourquie, 2004). However we need to note that we did not find a gradient of *FGF8* mRNA from our *in situ* on stage 23 and 27 embryo. Thus if in the growth factor screen *FGF8* was indeed found to be involved in endoderm specification it is unlikely that it acts through this mechanism.

BMP4 also showed an interesting expression pattern in both the anterior and posterior of stage 23 and 27 embryo. Although from the whole mount *in situ* it appears to be expressed in both endoderm and mesoderm, transverse histological section have shown that it is only expressed in the mesoderm. In chick *BMP4* was also found to be expressed in mesoderm around the gut but only near the posterior of the embryo (Roberts et al., 1995; Roberts et al., 1998), consistent with its ability ito inducen inducing a posterior respecification in chick endoderm and mesoderm culture (Kumar et al., 2003). The expression of *BMP4* in both anterior and posterior extremes of stage 23 and 27 *Xenopus* embryos might suggest that *BMP4* could be acting through a different mechanism in *Xenopus* and not just posteriorising the endoderm as previously seen in chick.

However there is also the possibility that the expression domain of *BMP4* might not have any relevance at all to the specification of the endoderm. The development of other organs has been shown to be dependent of the *BMP4* signal. The ventral foregut endoderm has the potential to develop into either liver or pancreas (Deutsch et al., 2001). Studies in mouse and chick have shown that the liver fate is dependent on signals from adjacent mesoderm: FGF signalling from adjacent cardiogenic mesoderm (Fukuda-Taira, 1981; Jung et al., 1999; Le Douarin, 1975); BMP from nearby septum transversum mesenchyme (Rossi et al., 2001); as well as an unidentified third signal coming from endothelial cells (Matsumoto et al., 2001). In *Xenopus* only *FGF* signalling have been implicated in liver development as a dominant negative inhibitor of *FGF* has been shown to inhibit induction of liver specific genes (Chen et al., 2003). Even though no

link has been made in *Xenopus* between *BMP4* and liver development, the anterior domain observed in our in situ is well placed to signal to the ventral foregut endoderm and induce liver fate.

The development of tail in *Xenopus* have also been shown to involve *BMP4*. Previous experiments in our lab have shown that ectopic *BMP4* expression in the posterior neural plate of early neurula embryo results in the formation of tail-like structure (Beck et al., 2001). It is also known that *BMP4* is expressed in the cells immediately ventral to the site of the future tail bud (Fainsod et al., 1994), and that it is active in the ventral part of the future tail bud itself which is fated to form tail somites (Beck et al., 2001). It is thought that *BMP4* is involved from an early stage in tail bud formation, as it is being determined (Beck et al., 2001). However the expression we see in our in situs does not show expression in the tail-bud and is slightly too anterior. Perhaps if we had used later stages embryos or developed for longer we might be able to see this tail bud expression.

Despite all these possible roles in liver and tail formation, we need to remember that *BMP4* was found to be expressed at the stages when endoderm specification occurs. Thus it is still possible that it is involved in the specification of the endoderm as well as the development of liver and tail. The functional role of *BMP4* as well as other growth factors in endoderm specification is something that we aimed to investigate in the growth factor screen in the next chapter

IV. Identifying mesodermal signals

IV.1. Introduction

Early studies on *Xenopus* endoderm development have suggested that it undergoes regional specification early in development and it does so autonomously. Expression of endodermal markers of development such as *Xlhbox8* and *IFABP* were found in vegetal explants isolated from blastula stage embryos (Gamer and Wright, 1995; Henry et al., 1996; Zorn et al., 1999). However a recent study in our lab has shown that this apparently autonomous specification of the endoderm in *Xenopus* is most likely due to the presence of mesoderm in these vegetal explants, which was previously missed due to the use of inappropriate mesodermal markers (Horb and Slack, 2001). The early studies in endoderm development had screened for *Xbra, cardiac actin, Xtwist* and *α-T_3 globin* to show that there was no mesoderm in the vegetal explants (Gamer and Wright, 1995; Henry et al., 1996; Zorn et al., 1999). However these genes were not expressed in the mesoderm surrounding the gut at tadpole stages. For the purpose of detecting mesoderm in the vegetal explants it is more appropriate to use mesodermal markers that are found in the gut mesoderm including those such as *FoxF1* (Koster et al., 1999),*XNkx-2.5* (Evans et al., 1995; Patterson et al., 2000; Smith et al., 2000), *XTbx5* (Horb and Thomsen, 1999) and *xFOG* (Deconinck et al., 2000). Expression of these was, in fact, evident in the vegetal explants (Horb and Slack, 2001), although whether this is due to dissection error, or to some regulative response of isolated endoderm is still not clear.

(Horb and Slack, 2001) in their study showed that mesoderm-free endoderm explants can only be made from later stage (stage 20-23) embryos, when the endoderm and mesoderm have formed distinct layers. These endodermal explants remain unspecified when cultured on their own. Expression of *Xlhbox8* and *Xcad2* were only seen if the endoderm explants were cultured in the presence of mesoderm. We need to note, however, that

in normal intact embryos *Xlhbox8* expression begins in late gastrula or early neurula. However Horb and Slack showed that this expression was lost in the absence of mesoderm, indicating that there must be a continuous requirement for mesoderm to maintain *Xlhbox8* expression in its normal territory. Only endoderms made from after stage 25 were able to maintain *Xlhbox8* expression in the absence of mesoderm. These observations suggested that the regional specification of the endoderm requires signalling from the mesoderm and is likely to occur later in development, becoming stable around stage 25 (Horb and Slack, 2001). This model for the regional specification of the *Xenopus* endoderm, where the mesoderm is essential, is similar to those in other organism such as mouse (Wells and Melton, 2000) and chick (Kumar et al., 2003).

In *Xenopus* only the instructive nature of the mesodermal signals have so far been characterised. Through heterologous recombinations, It was shown that posterior endoderm takes on an anterior identity when recombined with anterior mesoderm. Similarly anterior endoderm takes on posterior identity when recombined with posterior mesoderm thus indicating that the mesoderm is instructing the endodermal fate (Horb and Slack, 2001). The present study was intended to follow up from this and perform a screen to try and find the identity of the mesodermal signals being sent to the endoderm.

Simultaneous with this work, similar screens have been carried out in mouse (Wells and Melton, 2000) and chick (Kumar et al., 2003). In mouse it was found that FGF4 induces endodermal explants made from E7.5 embryo in a dose dependent manner. High doses of FGF4 were found to induce expression of *somatostatin* a posterior marker of development. Lower doses of FGF4 induced *NeuroD*, a more anterior marker of development whilst also downregulating *somatostatin* (Wells and Melton, 2000). Considering that *In vivo* FGF4 is normally expressed in the posterior of the embryo (primitive streak) (Niswander and Martin, 1992), these results indicate that

FGF4 might be acting as a posterior morphogen in patterning the endoderm.

In chick, FGF4 was also seen to pattern the endoderm in a posterior manner. FGF4 heparin-agarose beads implanted in the anterior most endoderm were seen to repress expression of the anterior markers *Hex1* and *Nkx2.1* whilst expression of the more posterior markers *Pdx1* and *CdxA* was upregulated. A complementary inhibitor study using SU5402 (FGFR1 inhibitor) heparin-agarose beads showed the opposite effect. Here *Hex1* expression was expanded whilst *Pdx1* was inhibited (Dessimoz et al., 2006). Another study in chick found that other growth factors such as RA, BMP and activin can induce respecification of the endoderm in the presence of mesoderm (Kumar et al., 2003). Here endoderm-mesoderm explants were induced to express more posterior specification markers when treated with these growth factors. It is worth noting that the patterning by growth factors in chick have not been demonstrated to be dose dependant.

In our study the growth factor screen would be based on the stage 20-23 endoderm explant culture that has been established in (Horb and Slack, 2001). In the screen, these explants would be treated with various growth factors and tested for expression of the specification markers *Xlhbox8* (anterior) and *Xcad2* (posterior) with either *in situ* or PCR.

Since the signals from the mesoderm have been shown to be instructive we expected to find expression of the *Xlhbox8* throughout the whole endoderm explant when treated with an anteriorising growth factor. Similarly we expected expression of *Xcad2* throughout the whole endoderm explant when treated with a posteriorising growth factor. Since in chick some growth factors only elicit a response in the presence of mesoderm we decided to perform a screen on explants where the mesoderm was cultured alongside the endoderm (endo+meso). A complementary inhibitor screen was also performed alongside the growth factor screen to provide a more

complete picture. A summary of the expected result from this screen can be seen on Figure 4.1 below.

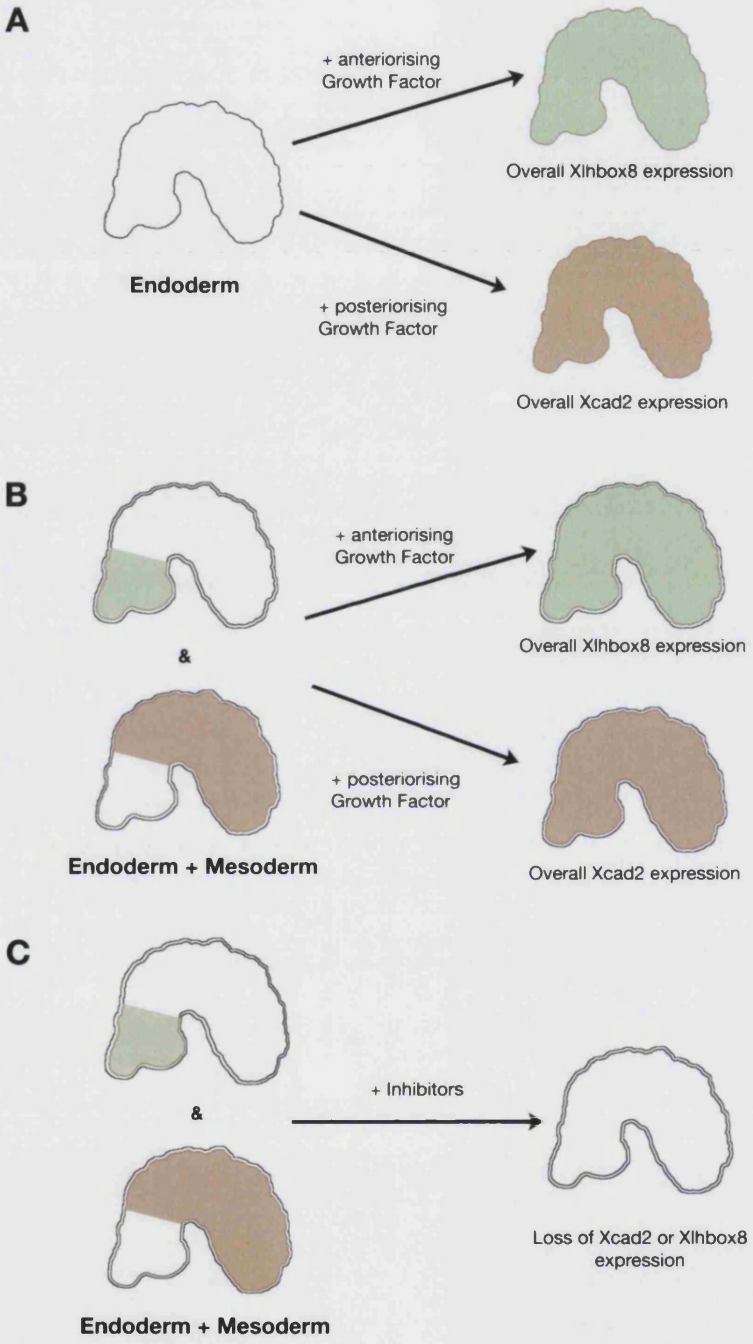


Figure 4.1 Summary of Hypothesis.
A) Endoderm-only screen. Untreated endoderm explant does not express either *Xlhbox8* or *Xcad2*. B) & C) Endoderm+Mesoderm screen. Untreated endo+meso explants show expression of *Xlhbox8* in the anterior and *Xcad2* in the posterior. Overall *Xlhbox8* expression was expected in both types of explant when treated with anteriorising growth factor whilst overall *Xcad2* was expected for posteriorising growth factors. Inhibitors that inhibit mesodermal signals should cause loss of expression of *Xlhbox8* or *Xcad2* in endo+meso explants.

IV.2. Results

Before going on with the growth factor screen we needed to optimise the detection methods to make sure that they could cope with the relatively small explants. PCR and *in situ* were the two methods used for the detection of specification markers. We explored the advantages and disadvantages of each of these techniques to determine a suitable detection method for the growth factor screen.

IV.2.1. PCR

RT-PCR was performed on RNA isolated from whole embryos of stage 10,18 and 27 for *Xlhbox8* and *Xcad2* (Fig 4.2). Here we saw a weak expression of *Xlhbox8* at stage 10 which increased through to stage 27 as was previously seen in (Horb and Slack, 2001). *Xcad2* expression was high throughout these stages, because it is expressed in mesoderm as well as the endoderm (Pownall et al., 1996).

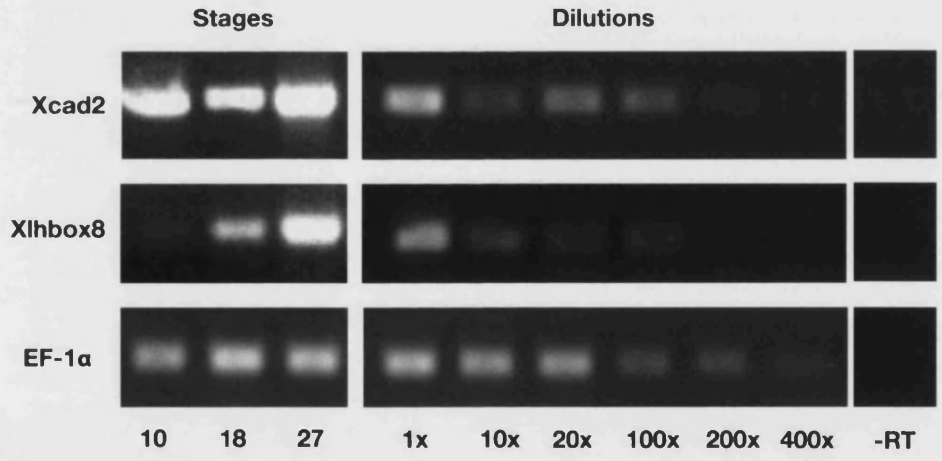


Figure 4.2 RT-PCR with whole embryos.
RT-PCR result for *Xcad2* and *Xlhbox8* for stage 10.18 and 27 are shown on the left. RNA samples from stage 27 was then diluted for 1x,10x, 20x, 100x, 200x and 400x to test the limits of RT-PCR detection. Results of this dilutions are shown to the right. EF-1α was used as a positive control.

αSince the screening was to be done on smaller pieces of tissue we wanted to see how far we could extend the sensitivity of the RT-PCR detection. To do this RNA was isolated from a single stage 27 embryo and

was put through a dilution series up to 1 in 400 before being reverse transcribed (Fig 4.2). The result showed that for *Xcad2* detection was still possible up to a dilution of 1 in 200 with *Xlhbox8* being slightly less sensitive and detectable up to 1 in 100 dilution. The very high sensitivity of this method means that it should be possible to detect expression of both these genes from small tissue explants..

Previously in (Horb and Slack, 2001) RT-PCR detection was done on half endoderm samples, dividing the whole endoderm into anterior and posterior pieces. The establishment of this high threshold of RT-PCR detection meant that we could further divide the endoderm and thereby increase the spatial resolution of this method of detection. Thus, we decided on dividing the endoderm into 4 regions along its A-P axis into Extreme Anterior (EA), Anterior (A), Posterior (P) and Extreme Posterior (EP) pieces (Fig 4.3).

Figure 4.3 Explant diagram and RT-PCR.
The diagram on the left shows how the regions of the endoderm were divided along the A-P axis. This was explanted from stage 23 endoderm cultured and then processed for RT-PCR. Result for this are shown on the right with Gut from stage 42 was used as positive control.

Endo+meso explants of these regions were then made and cultured until the control reached stage 42. Note that in making the endo+meso explants the ectoderm was not removed. Even though the PCR can detect up to 1 in 100 dilution of RNA we found that the RNA extraction step was less efficient for small explants and so at least 20 pieces of each region is necessary for good RNA extraction. For a positive control the complete gut

from the stage 42 embryo was used. This gut represents a normally developed endoderm tissue and gives a representation for the normal expression levels of *Xlhbox8* and *Xcad2* in the endoderm. 5 whole guts were collected and then processed as a positive control. Results of the PCR on cultured explants and dissected gut are shown in Fig 4.4.

From this RT-PCR we found that *Xcad2* was expressed throughout all four regions, with a slightly higher level seen at the EP region. *Xlhbox8* on the other hand was expressed only in the anterior pieces with EA expression being slightly higher. These results showed that the spatial resolution of the PCR was not increased dramatically by increasing the number of explants made from the endoderm from 2 to 4.

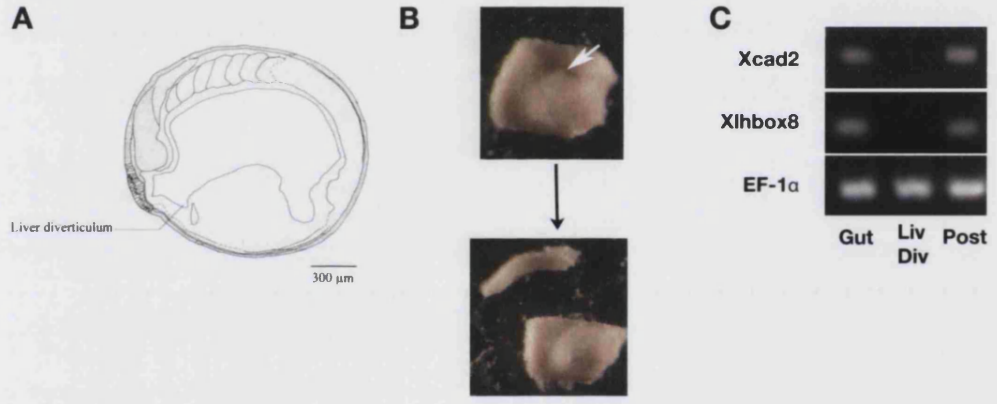


Figure 4.4 Liver diverticulum explants.
A) Diagram from a parasagital section of stage 20 endoderm showing the liver diverticulum. B) White arrow on the top photo shows the position of the liver diverticulum on whole endoderm. The resulting explants generated from this cut is shown on the bottom photos. C) RT-PCR result for these explants with stage 42 gut used as control. Expression of Xcad2 or Xlhbox8 is contained in the posterior piece (Post). No expression was seen in the anterior piece (Liv Div).

RT-PCR on the explants also showed the lack of an *Xlhbox8*-only expressing region which would be advantageous for detecting posterior shifts in identity. To address this we tried a more refined cut of the endoderm. Between stage 20-23 of the endoderm development the liver diverticulum is a visible small indentation in the endoderm. By cutting the endoderm at this point we could produce a smaller anterior piece than the

EA region described in Fig 4.4, which should hopefully remove the part that expresses *Xcad2*. The diagram on Fig 4.4 shows the liver diverticulum (Fig 4.4A) and the explants created as a result of a cut at this part of the endoderm (Fig4.4B). 20 of the small anterior (Liv Div) and larger posterior (Post) explant pieces were collected and processed by RT-PCR for *Xlhbox8* and *Xcad2* (Fig 4.4 C).

The result shows that the Liv Div explants contain no expression of either *Xlhbox8* or *Xcad2*. It would seem that the expression of *Xlhbox8* and *Xcad2* was from tissue posterior to the liver diverticulum. The *EF-1α* expression in Liv Div shows that the lack of *Xlhbox8* and *Xcad2* expression was probably a real result and not an artifact.

IV.2.2. *In situ*

Three probes were made for the *in situ* hybridisation: *Xlhbox8*, *Xcad2* and *Edd*. There were no problems in generating DIG-RNA probes for *Edd*. This probe when tested with gut isolated from stage 41-43 embryo gives an expression pattern that covers the entire gut with the exception of liver (Fig 4.5A). Although there is a period when *Edd* is ubiquitous in the endoderm, by this stage of development expression of *Edd* has been turned off in the liver.

On the other hand, initial attempts to generate a DIG-RNA probes for *Xcad2* and *Xlhbox8* from linearised plasmid template transcribed it with T7 polymerase was unsuccessful. The transcription reaction yielded no detectable RNA. This was probably because the constructs for *Xlhbox8* and *Xcad2* were in pCS2+ which has a mutated T7 promoter. Our first attempt to solve this problem was by using PCR. PCR primers that flanked the gene construct were made, one just before the polylinker and another at the T7 promoter. The primer at the T7 promoter was modified so that it contained the normal T7 sequence to subtitute for the mutated T7 promoter in the plasmid (see Section 2.4.1, p41). Thus using this primer pair, we can obtain PCR products that have a normal T7 promoter and the appropriate gene

construct suitable to be used in a transcription reaction to produce RNA. We were able to obtain DIG-RNA probes for both *Xlhbox8* and *Xcad2* using this method. However only the *Xlhbox8* probes generated with this method consistently gave an anterior expression pattern on stage 41-43 gut (Fig 4.5A). *Xcad2* was more inconsistent, on average only 1 in 4 transcription reactions would give rise to the posterior expression pattern on stage 41-43 gut (Fig 4.5B).

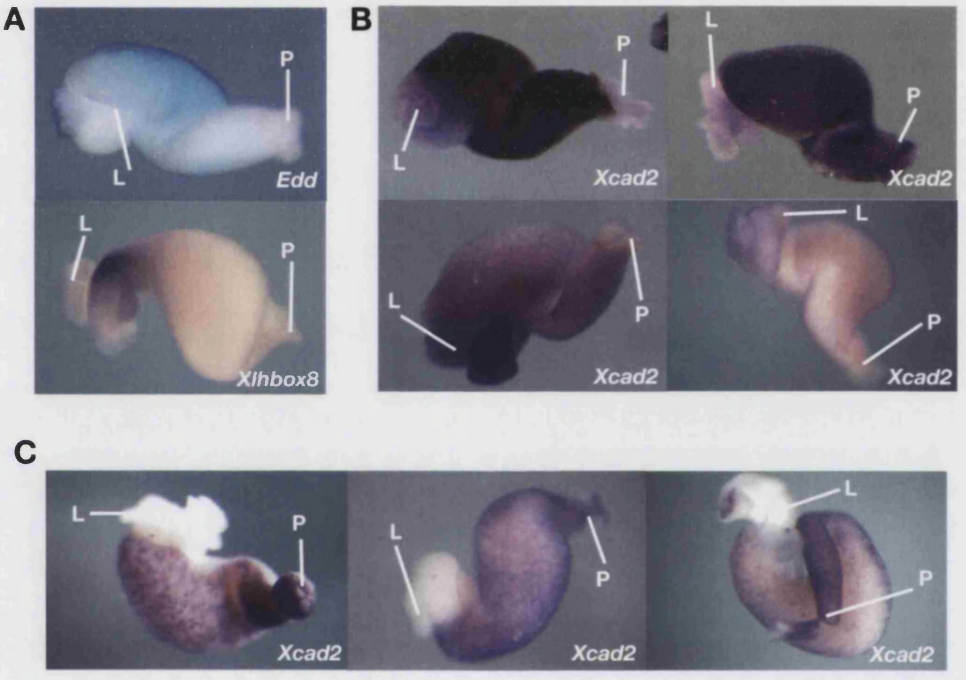


Figure 4.5 Control *in situ*s.
All *in situ*s were performed on whole gut isolated from stage 41-43 embryo. To help orientate the samples the positions of Liver bud (L) and proctodeum (P) are highlighted in the panels. A) shows the expression pattern for *Edd* and *Xlhbox8* on gut. *Edd* was expressed throughout most of the gut with exception of liver, whilst *Xlhbox8* was only expressed in the anterior B) shows the expression pattern of *Xcad2* DIG-RNA made from pCS2 construct on gut. Only 1 in 4 gave a correct pattern where the anterior of the gut was not stained. C) the expression pattern of Xcad2 DIG-RNA made from pBS construct. Results from this construct were consistent, showing posterior expression domain for *Xcad2.*

We tested our stock *Xcad2* plasmid for possible contamination and found that it had been contaminated with empty pCS2+ plasmid. This empty pCS2+ plasmid would have resulted in the production of non-specific DIG-RNA probes during the transcription reaction that would explain the

inconsistencies observed in the *in situs*. We later obtained an *Xcad2* construct from Harv Isaacs that was in pBS which has a normal T7 promoter. DIG-RNA probes made from this construct were able to give consistent posterior expression pattern for *Xcad2* (Fig 4.5C).

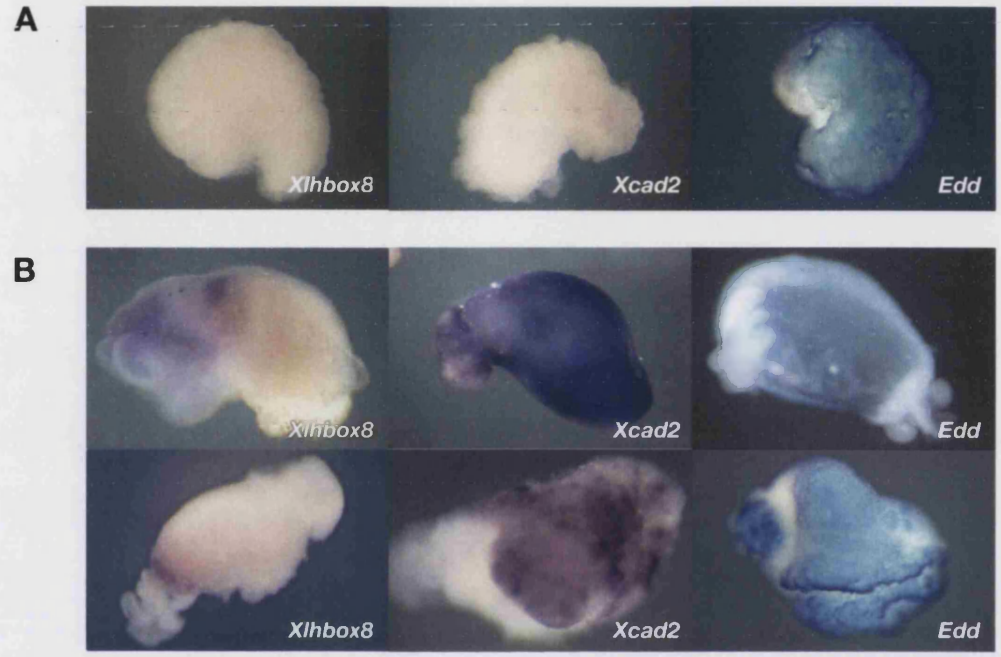


Figure 4.6 *In situ* on explants.
A). Ahows *in situ* done for *Xlhbox8*, *Xcad2* and *Edd* on endoderm-only explanta. No expression of *Xlhbox8* or *Xcad2* was seen B). Shows *in situ* done for *Xlhbox8*, *Xcad2* and *Edd* on endo+meso explants. In the top row the 'bubble' possible from the ectoderm surrounding the explant was not removed. *Xcad2* photo shows retention of BM purple and dark colours. Bottow row shows *in situ* where explants have had the 'bubble' removed showing clearer staining. An unstained anterior part was visible in the *Xcad2 in situ*.

Having established consistent and reliable probes for the genes *Edd*, *Xlhbox8* and *Xcad2*, we moved on to test expression patterns on explants. Whole endoderm and endo+meso explants were made from stage 20-23 embryo and cultured *in vitro* until control embryos reached stage 41. Figure 4.6 shows the expression pattern of *Xlhbox8*, *Xcad2* and *Edd* on these explants. These results were the same as previously reported in (Horb and Slack, 2001) where endoderm cultured in the absence of mesoderm showed no expression of the specification markers *Xlhbox8* or *Xcad2* (Fig 4.6A). This was not due to the explant dying as *Edd* was still expressed. With

endo+meso we saw expression of *Xlhbox8* in the anterior and *Xcad2* in the posterior indicating normal specification of the endoderm (Fig 4.6B)

The surrounding ectoderm that were present in endo+meso explants is thought to form a bubble of epidermis surrounding the explant during the culture period. This bubble may cause problems during the *in situ* development as it seemed to retain the BM-purple substrate used in colour development. This was most evident in the *Xcad2 in situ* in Fig 4.6B top row. Although not evident in the *in situs* we have performed so far, retention of the substrate also brings up the possibility of false positives and misrepresentation of expression domains. To avoid this we removed the bubbles surrounding the explants prior to fixation using forceps. This resulted in better and clearer staining of the explants as can be seen when we compare *in situ*s done on endo+meso explants with and without the "bubble" (Fig4.6B). *Xcad2* staining in particularly better as it now revealed an unstained anterior domain in the endo+meso explant where before it may have appeared to be expressed on the whole explant.

IV.2.3. Screen strategy

We have seen that both PCR and in situ can be used to detect the specification markers *Xlhbox8* and *Xcad2* in explants. However for the purpose of our screen we decided that *in situ* was more appropriate. Detection of specification and shifts in expression domain requires spatial resolution which is more evident in the *in situ*s compared to PCRs. Also since the *in situ*s do not require the endoderm to be divided into separate regions to obtain the spatial resolution, it would make for a simpler and quicker screening method.

The screen was performed on whole endoderm and endo+meso explants from stage 20-23 embryo. These explants were then cultured with or without growth factor and inhibitor until the control embryos reached stage 41. At this point the explants were fixed and processed with *in situ* hybridisation for *Xlhbox8*, *Xcad2* and *Edd.* Table 4.1 lists the growth factors

and inhibitors to be tested along with the concentration they were tested at. Growth factors were selected to encompass the major pathways in embryonic development: FGFs (FGF4, 8 and 10), BMP (BMP4), TGF-ß (ActivinA), Wnt (LiCl), EGF and RA. LiCl was used to mimic the Wnt pathway as the Wnt protein was not commercially available. LiCl mimics activation by Wnt by inhibiting the action of Gsk3ß leading to accumulation of ß-catenin (Klein and Melton, 1996; Schneider et al., 1996). In our LiCl treatments we used a modified NAM solution where the the NaCl salt were replaced with LiCl. Embryos were treated for 30 mins, an hour or overnight with this solution to mimic Wnt activation.

Table 4.1 Growth Factor and Inhibitor list

Endoderm	**Endoderm +Mesoderm**	
Growth Factor	**Growth Factor**	**Inhibitor**
Activin A (2 & 20ng/ml)	Activin A (2 & 20ng/ml)	Cyclopamine (10 & 100 ng/ml)
BMP4 (50 & 500ng/ml)	BMP4 (50 & 500ng/ml)	EGF/EFRB-2 (10 & 100 ng/ml|)
FGF4 (10 & 100ng/ml)	FGF4 (10 & 100ng/ml)	Follistatin (10 & 100ng/ml)
FGF8 (10 & 100ng/ml)	FGF8 (100ng/ml)	sFRP-2 (10 & 100ng/ml)
FGF10 (10 & 100ng/ml)	FGF10 (10 & 100ng/ml)	Noggin (10 & 100ng/ml)
Egf (10 & 100ng/ml)	Egf (10 & 100ng/ml)	PD 98059 (100ng/ml)
*LiCl (in NAM 30 min, 1hr and overnight)	*LiCl (in NAM 30 min, 1hr and overnight)	SU5402 (5 & 10 µM)
RA (10^{-4} to 10^{-7} M)	RA (10^{-4} to 10^{-7} M)	

* Note that LiCl is not a growth factor and is listed here as a subtitute for WNT.

For the complementary study, we chose inhibitors that would block the pathways that were tested in the growth factor screen. Follistatin is an Activin antagonist, sFRP-2 inhibits Wnt; noggin inhibits the BMP pathway (Dale and Jones, 1999; Delaune et al., 2005; Slack and Tannahill, 1993); PD98059 is a MAPK kinase inhibitor; SU5402 inhibits FGFR1 binding

(Chung et al., 2004; Delaune et al., 2005); EGF/EFRB-2 inhibits activity of Egf; and cyclopamine inhibits *Sonic hedgehog (Shh)*.

The concentrations at which these growth factors and inhibitors were tested was set arbitrarily. This was because that even though some of the growth factors and inhibitors have been tested in *Xenopus* previously they have not been tested on the endoderm and endo+meso explants exclusively. Thus we decided to have two concentrations tested, a low and a high concentration for each growth factor.

IV.2.4. Growth Factor Screen - Positive control

The activity of the growth factors were tested alongside the explant experiment to ensure that they were active under the conditions used, and not, for example inactive because of absorption onto the vessels. The methods for the positive controls differed depending on the growth factor (Figure 4.7). The FGFs and ActivinA were tested using the animal cap assay. Addition of ActivinA (Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990) has been shown to induce mesoderm formation and elongation of animal caps. FGF4 (XeFGF in *Xenopus*) (Isaacs et al., 1992b), FGF8 (Christen and Slack, 1997) and FGF10 have been shown to induce formation of mesoderm in animal caps indicated by the formation of vesicles (Godsave et al., 1988; Slack et al., 1987). For our animal cap assay we used a concentration of 1 ng/ml for ActivinA and 100ng/ml for FGF4, 8 and 10.

FGF8 mRNA is also a very potent posteriorising factor when injected into embryos (Christen and Slack, 1997). Therefore FGF8 function was also tested by injecting 500pg of FGF protein into blastula stage embryo and observing posteriorisation of the embryo. BMP4 was also tested in this way. Injection of BMP4 mRNA into blastula stage embryo has been shown to produce ventralised embryos (Dale et al., 1992; Fainsod et al., 1994; Jones et al., 1992). EGF protein was tested by treating fertilised embryos with 200 ng/ml of the protein. Approximately 30% of the embryo shows posteriorisation of the embryo similar to that of FGF8 protein injection.

RA and LiCl were tested by treating whole embryos. These three substances have been shown to disrupt normal development of the embryo when applied to pre-gastrulation embryos. RA is known to inhibit head formation when put in the culture medium (Eagleson et al., 2001) whilst LiCl provokes dorsalisation (Klein and Melton, 1996; Schneider et al., 1996). RA was put on in the medium at 10^{-4}M overnight and LiCl (0.1M substituting for Na in NAM) for 30 mins and an hour (overnight treatment killed the embryos). Figure 4.7 shows the results of these positive control tests on the different growth factors. These showed that the growth factor proteins and other substances were biologically active under the conditions used.

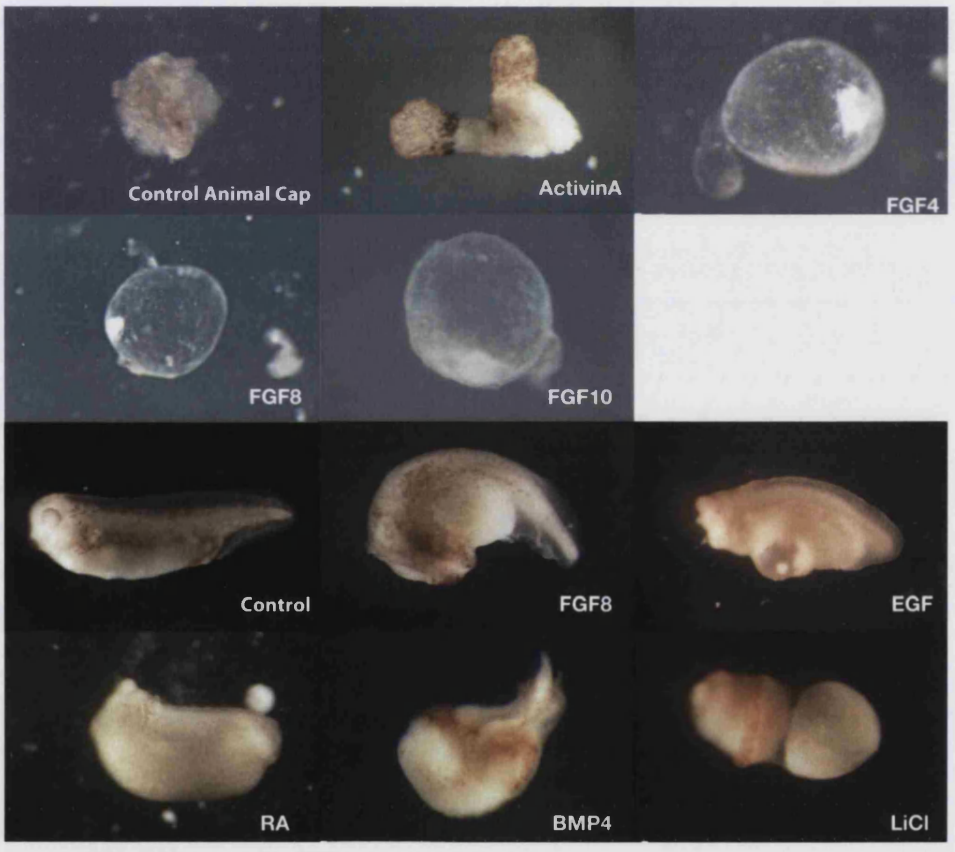


Figure 4.7 Positive controls for growth factors.
The photographs above show result of activity assays done for the various growth factors. Control animal cap and stage 32 embryo were untreated. ActivinA treated animal cap showed clear elongation. An expansion of the animal cap was seen when FGF4, 8 and 10 were put on the animal caps indicating mesoderm induction. FGF8 also induces posteriorisation when injected onto embryos. EGF also has a similar posteriorising effect. RA produces a shortened trunk and head suppression. BMP4 ventralises the embryo and LiCl dorsalises them.

The positive controls described above did not address whether the growth factors have the expected biochemical effect on the explants. We have attempted to create an assay based on western blots to detect directly whether the growth factors did activate their respective signalling pathways on the explant. However there was a difficulty in obtaining good blots especially with phosphorylated proteins such as p-Erk. At early stages most of the embryo consists of yolk proteins and these usually cause problems in loading and running the gel and must be removed. Also, most of the commercially available antibodies were not designed for the *Xenopus* protein, making detection of phosphorylated protein that has a very small antigenic determinant even more difficult. Without the ability to detect the phosphorylated proteins reliably, we would not be able to tell whether the pathways were activated or not in the explants. Thus the western blot method was not pursued further.

IV.2.5. Endoderm growth factor screen - *in situ* hybridisation

Here we present the result from our first screen, the treatment of growth factors on mesoderm free endoderm explants with growth factors. Each treatment was performed in triplicate on 60 endoderm explants for each replication. This was to ensure that results were reproducible and that a significant number of explants would be available for the in *situ* hybridisation (20 per probe). On average approximately 90-100% of the growth factor treated explants survived the length of the culture period with one exception. LiCl treatments overnight resulted in the death of the explants. Figure 4.8 on the next page shows the *in situ* results done on the treated explants. The explants shown in this figure were those treated with the highest concentration of growth factor listed in Table 4.1 as results were similar with treatment at the lower concentrations (data not shown).

We observed no expression of *Xlhbox8* or *Xcad2* in any of the treatments. The *in situ* pattern of these treated explants were similar to untreated explants (Fig 4.8). Because *Edd* was still expressed, the missing *Xlhbox8* and *Xcad2* expression were probably indicative that the endoderm

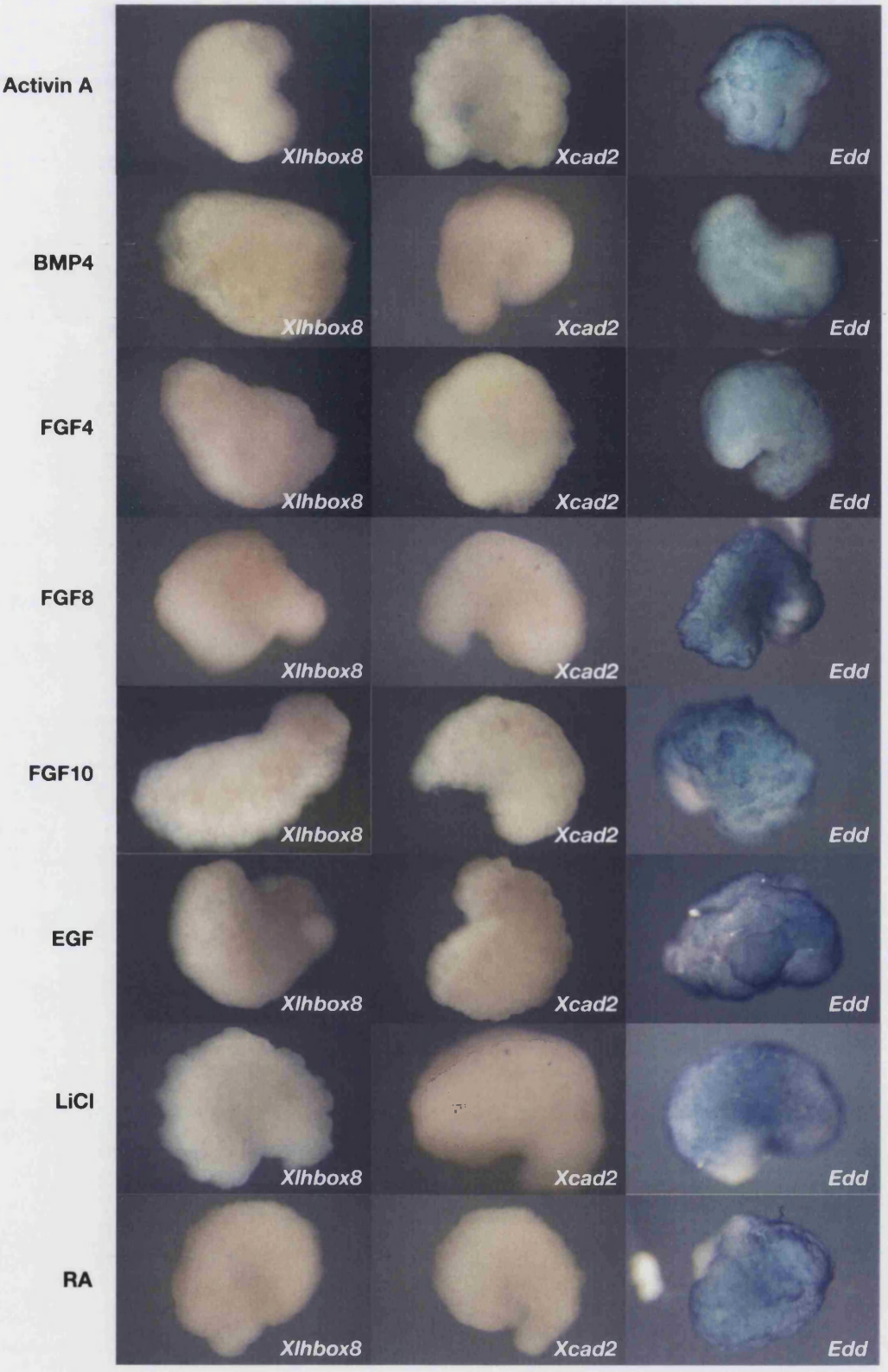
was still unspecified. In other words none of the growth factors tested were able to mimic or replace signals being sent by the mesoderm during regional specification of the endoderm.

IV.2.6. Endoderm + Mesoderm growth factor screen - *in situ* hybridisation

Similar to the endoderm explant growth factor screen, the screen on endo+meso explants was performed in triplicates for each treatment with 60 explants per treatment. With endo+meso explants the survival rate was very close to 100% with one exception, the overnight treatment with LiCl. The presence of mesoderm on these explants increased their viability compared to the endoderm-only explants an observation that was noted as well in mouse (Wells and Melton, 2000) and chick (Kumar et al., 2003).

Figure 4.9 shows the *in situ* results for the treated endo+meso explants with *Xlhbox8*, *Xcad2* and *Edd*. Here we observed that neither *Xlhbox8* or *Xcad2* expression had strayed from its normal expression domain. *Xlhbox8* was still found to be expressed in the anterior part of the explants whilst *Xcad2* covered the posterior part with a distinct, unstained anterior part. This pattern was very similar to that observed in untreated endo+meso explants (Fig 4.9) and shows that the growth factors had no effect on the specification of the endoderm in endo+meso explants.

Figure 4.8 *In situ* results on growth factor treated endoderm-only explants.
Results for *Xlhbox8*, *Xcad2* and *Edd* in situ on the different growth factors tested are shown on the next page. No expression of *Xlhbox8* or *Xcad2* was seen from any of the growth factor treatments. The expression of Edd seen in all the treatments indicates that the explants were still alive and that the lack of Xlhbox8 and Xcad2 expression was not due to the embryos dying.


Activin A
BMP4
FGF4
FGF8
FGF10
EGF
LiCl
RA
Xlhbox8
Xcad2
Edd

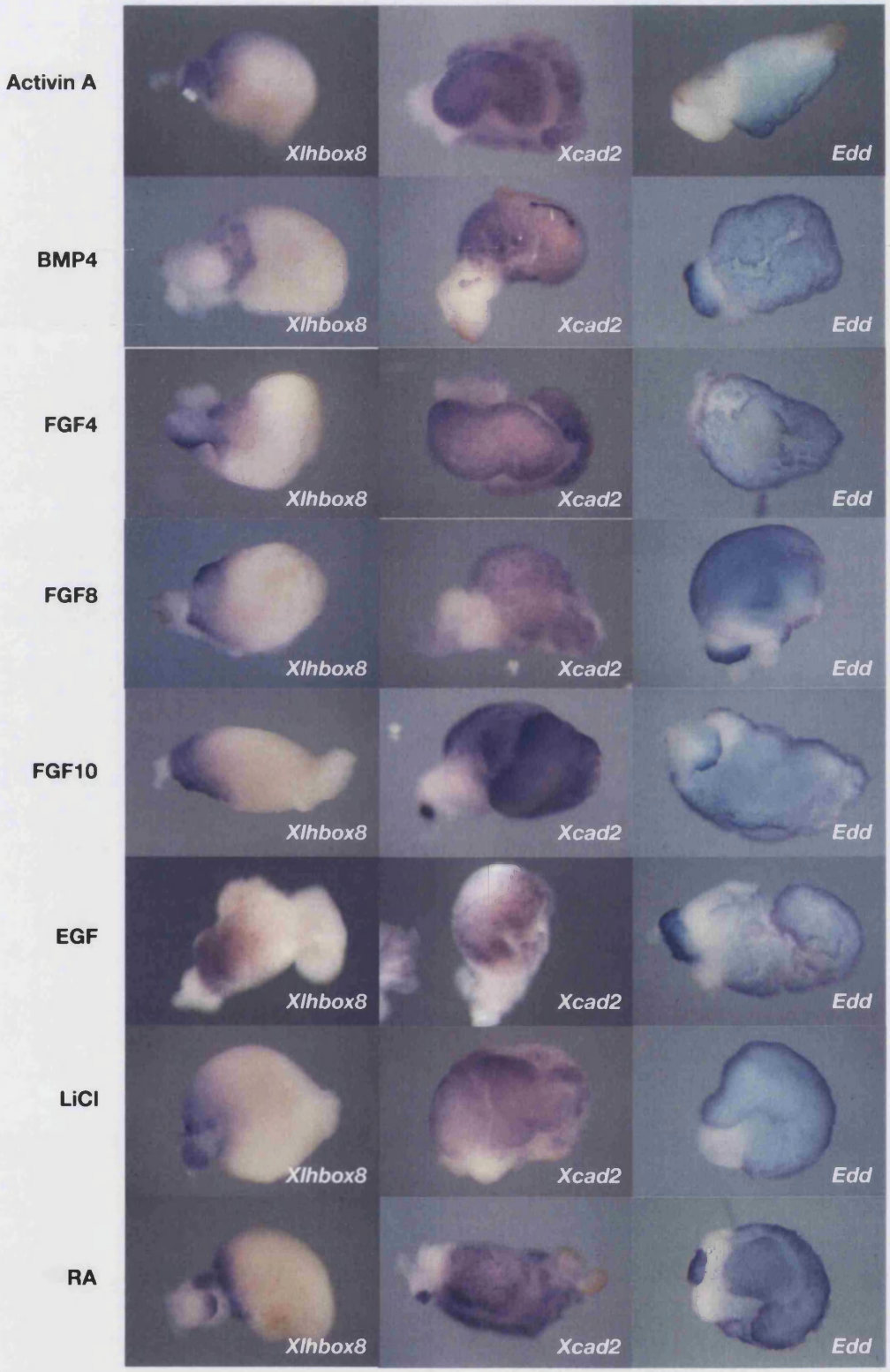


Figure 4.9 In situ results on growth factor treated endo+meso explants.
Results for *Xlhbox8*, *Xcad2* and *Edd* in situ on the different growth factors tested are shown above. Expression domains remained unchanged, *Xlhbox8* was still found in the anterior part of the explant whilst *Xcad2* still had a distinct unstained anterior section. Edd expression indicated that the explants were alive

IV.2.7. Inhibitor screen - positive control

Amongst the inhibitors tested in our experiments only noggin and SU5402 had a previously documented phenotype on *Xenopus* embryos, and in both cases we were able to reproduce this. SU5402 causes loss of axial structures on embryos treated from before gastrulation in a dose dependent manner (Delaune et al., 2005). When the embryos were treated with 10μM SU5402 we saw a bent tail phenotype in the embryos. Noggin has also been shown previously to act as a dorsalising factor in *Xenopus* (Dale and Jones, 1999; Delaune et al., 2005; Slack and Tannahill, 1993). 500pg of noggin protein was injected to blastula stage embryos and lead to dorsalisation of the embryo. In addition to these, the following new results were obtained for the other inhibitors. Embryos treated with cyclopamine (1 μg/ml) shows a curved axis, something that has not been previously described. The other 4 inhibitors tested EGF/EFRB-2, Follistatin, sFRP-2 and PD98059 did not show any visible phenotype when added at concentrations up to 1μg/ml.

For the inhibitors that have not been characterised previously in *Xenopus* we decided to treat whole embryos with the inhibitors at concentrations of 1 μg/ml. The lack of a visible phenotype with some of the inhibitors does not rule out a possible effect on the embryos as it might not be immediately obvious. We decided not to characterise the phenotype of each of the inhibitors as we were more interested in their possible effects on the endoderm. To mitigate the lack of a positive control phenotype with some of the inhibitors we used freshly bought preparations (<1 week).

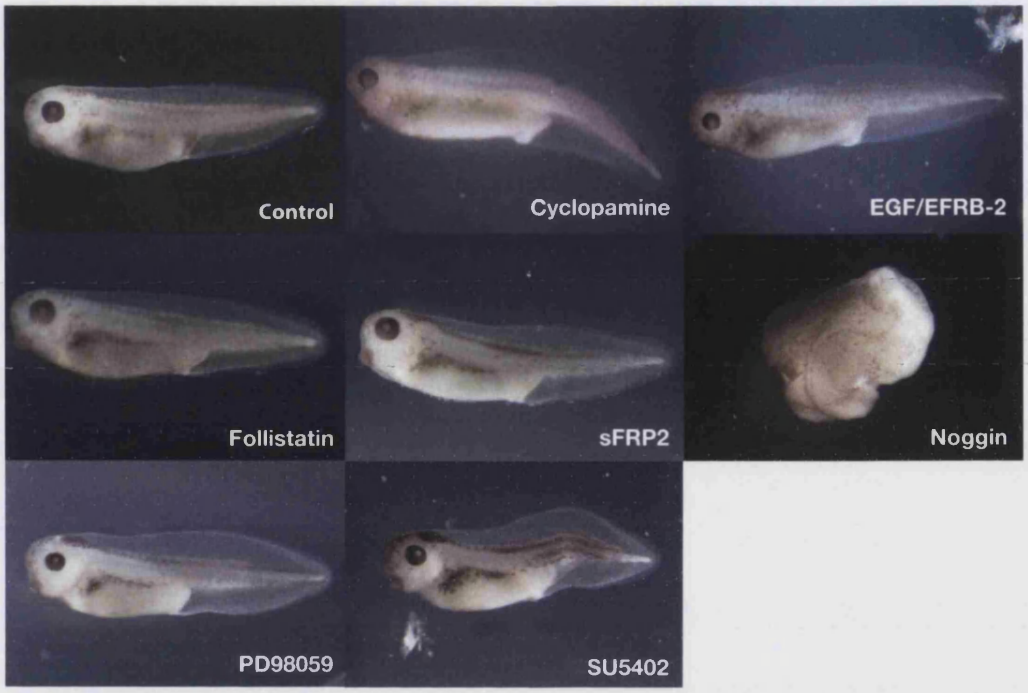


Fig 4.10 Inhibitors positive control.
The figures above shows the resulting phenotypes obtained from treating whole embryos prior to gastrulation with the inhibitors listed in Table 4.1. Cylopamine embryos has a curved axis. SU5402 showed a bent tail phenotype. Whilst Noggin injection resulted in dorsalisation of the embryo. EGF/EFRB-2, Follistatin, sFRP2 and PD98059 showed no phenotype at the concentrations tested.

Deciding what concentrations would be ideal for treating explants was difficult as we did not have a viable method for detecting downregulation of the target pathways in the explants. Thus we decided to base the concentrations we used on the positive control for those that had a clear or previously characterised phenotype. For those molecules that did not have a clear or previously characterised phenotype we decided on the arbitrary concentration of 100 and 10ng/ml to match the concentrations at which the growth factors was tested (For a list of concentrations used see Table 4.1).

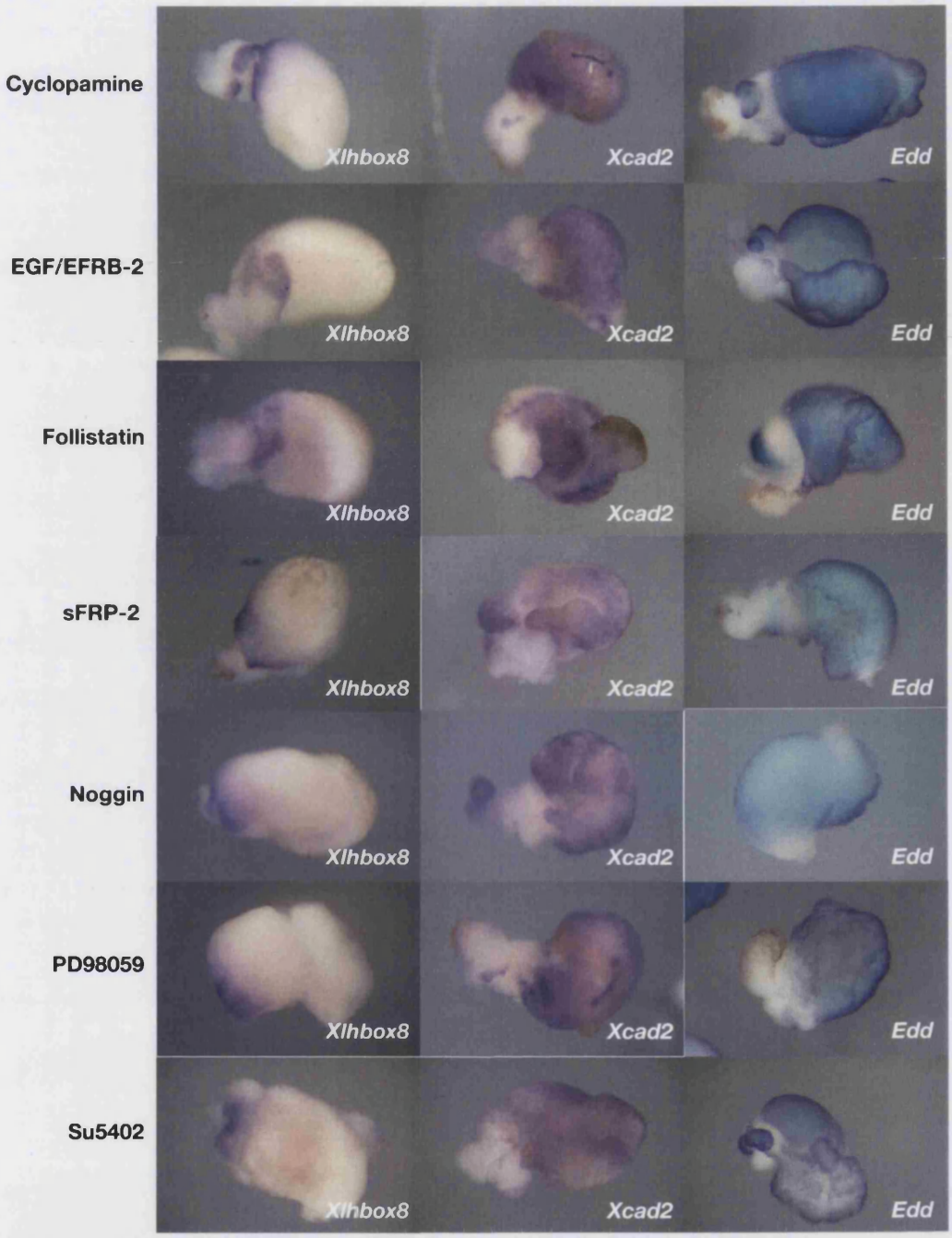


Figure 4.11 *In situ* results for inhibitor treated endo+meso explants.
Results for *Xlhbox8*, *Xcad2* and *Edd* in situ on the different inhibitors tested are shown above. Expression domains remain unchanged, *Xlhbox8* was still found in the anterior part of the explant whilst Xcad2 still had a distinct unstained anterior section. *Edd* staining confirmed that the embryos were alive.

IV.2.8. Endoderm + Mesoderm Inhibitor screen - *in situ* hybridisation

Again treatment of the endo+meso explants with inhibitors were done in triplicates with 60 explants per repetition. Survival of the explants was

close to 100%. Figure 4.11 shows in situ results done on these explants for *Xlhbox8*, *Xcad2* and *Edd*. All treatments was done in NAM/2 with 1% BSA as carrier.

We observed no changes in the expression domains of either *Xlhbox8* and *Xcad2*. *Xlhbox8* was still expressed in a tight anterior domain and *Xcad2* was expressed in the posterior domain with a distinct unstained anterior region. This result then complemented the results observed with the endoderm and endo+meso growth factor treatments where no change was seen when compared to untreated explants.

IV.2.9. Bead experiment

There was a possibility that the negative results observed with the growth factor and inhibitor screen was because the growth factor delivery method was not appropriate. The explant method delivered the growth factors *in vitro* at a specific concentration for a limited amount of time. However *in vivo* growth factor signals are usually continuously released and may form a gradient. We can mimic this by using heparin-acrylic beads, an in vivo method of delivering growth factors. FGFs are known to bind to heparin-acrylic beads (Slack et al., 1987). The heparin bound FGFs can then be implanted to the embryo to treat the endoderm *in vivo*, this implanted bead will then continuously release the growth factor throughout the culture period, mimicking more accurately *in vivo* signals. We decided to start the treatment with FGF4 as this had been shown to have a posteriorising effect in chick (Dessimoz et al., 2006) and mouse (Wells and Melton, 2000). To confirm that FGF4 can be bound to heparin-acrylic beads and was active, we performed an animal cap assay. Animal caps that were put in contact with the FGF4 beads showed elongation, while those put in contact with control PBS beads did not (Fig 4.12B). This was consistent with mesoderm induction caused by FGF induction (Godsave et al., 1988; Slack et al., 1987).

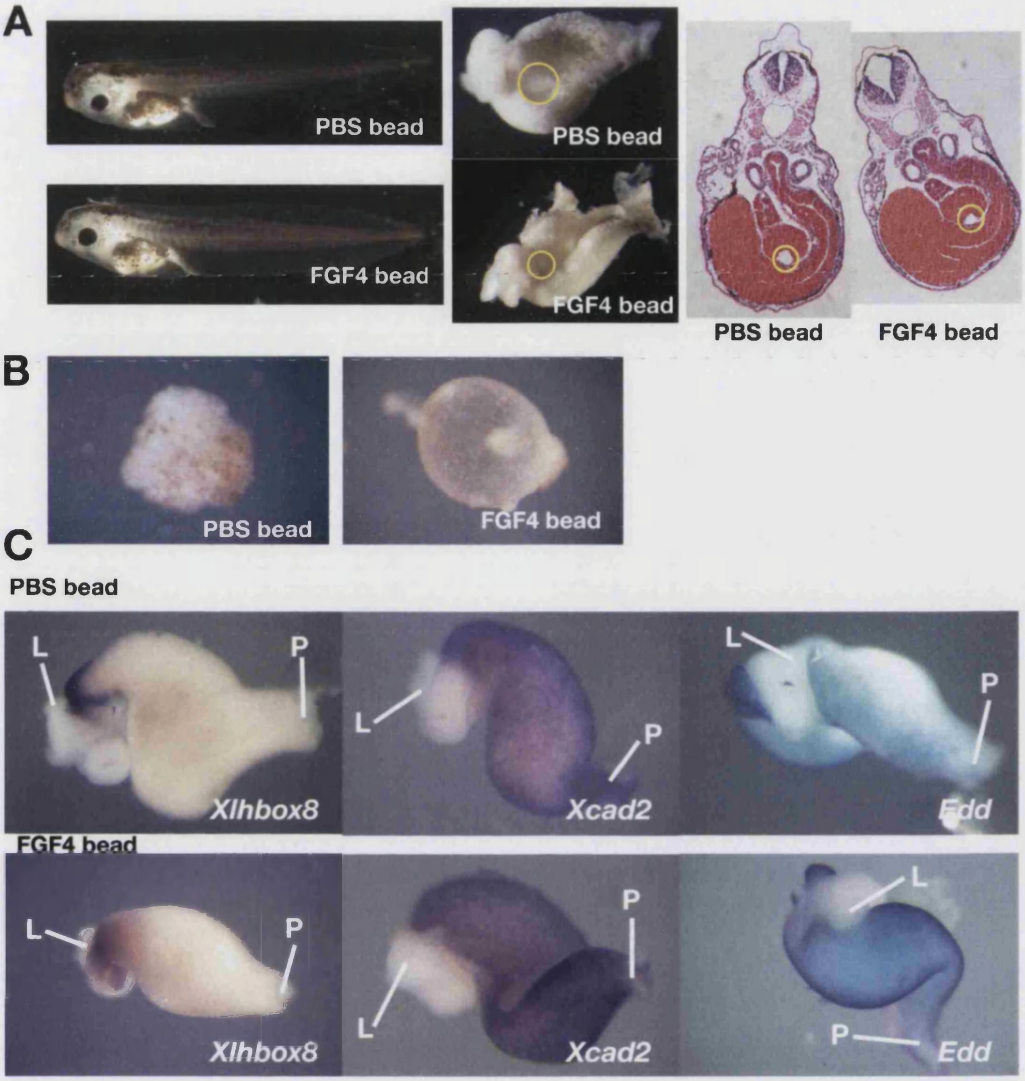


Figure 4.12 FGF4 bound heparin-acrylic beads treatment.
A) shows the typical appearance of bead treated embryos at stage 42. The presence of beads inside the embryos were confirmed by dissecting the gut and through histology. Position of the beads were highlighted by the yellow circle. B) Animal cap assay showing that the FGF4 incubated beads were capable of inducing mesoderm in animal caps whilst PBS beads were not. C) *in situ* results for *Xlhbox8*, *Xcad2* and *Edd* performed on guts isolated from bead treated embryos. To help orientate the positions of liver bud (L) and proctodeum (P) are highlighted in the panels.

After confirming the activity of the FGF4 beads, we went on to implant the beads into the anterior of stage 20-23 embryo. The embryos were allowed to heal and were cultured until stage 42. Once the embryos reached stage 42 their guts were dissected out and processed for in situ hybridisation for *Xlhbox8*, *Xcad2* and *Edd*. Since normally FGFs are

posteriorising agents (Christen and Slack, 1997; Isaacs et al., 1994) we expected this anterior placement would posteriorise the anterior of the *Xenopus* endoderm, inhibiting expression of *Xlhbox8* whilst promoting *Xcad2*.

The FGF4 bead-treated embryos at stage 42 looked similar to PBS bead-treated embryos, no abnormalities of the gut was observed (Fig 4.12A). *In situ* results further confirmed that there were no difference between gut of FGF4 bead treated embryos (Fig 4.12C). This was indicative that the lack of response seen in the in vitro screen for FGF4 was not due to the growth factor delivery method used as both beads and growth factor in media did not change the specification of the endoderm.

At this time we discovered that James Wells and his lab have attempted a similar FGF4 bead implantation in *Xenopus* embryos (unpublished results). They claimed that FGF4 bead implanted on the anterior of stage 15 embryos would lead to a bulging of the anterior of the embryo. This was an indication that endoderm development had been affected by the FGF4 beads although no attempt had been made to determine if specification was also altered.

Based on this we decided to expand our FGF4 bead experiment to include implantation done on stage 9, 10.5, 13 and 15 embryos. The beads were implanted in the embryos by making an incision in the anterior in stage 13 and 15 embryos or in the dorsal of stage 9 and 10.5 embryos and inserting the bead from the gap created. Once implanted the embryos were allowed to heal and were cultured until they reached stage 42. We found however that our results was not similar to James Wells' claim. From our experiments we saw that the FGF4 implanted embryos did not show any visible bulging or defect in the endoderm when implanted at stage 9, 10.5, 13 and 15. This was similar to when the beads were implanted in stage 20-23 embryos (data not shown).

IV.3. Discussion

All of the experiments described above showed that none of the growth factors tested can affect the specification of the endoderm, as expression patterns of either *Xlhbox8* or *Xcad2*, anterior and posterior markers of endoderm development remained similar to untreated controls in either endoderm or endo+meso explants. The growth factors were shown to be biologically active through the positive control experiments performed alongside the screen. Thus, based on this we have shown that there were no evidence tha the growth factors tested can influence the specification of the endoderm at the concentrations tested..

From all the growth factors tested a lack of respecification of the *Xenopus* endoderm by FGF4 is particularly interesting considering that in chick and in mouse it has been shown to affect specification of the endoderm (Dessimoz et al., 2006; Wells and Melton, 2000). From our previous *in situ* results (chapter III) we observed that FGF4 is expressed at the posterior of the endoderm, so at least it is expressed in a relevant region to act as a posteriorising agent. We have attempted both *in vitro* (explants) and *in vivo* (beads) approaches with this growth factor and still found no respecification, leading us to believe that FGF4 was not involved in endoderm specification.

We need to note, however, that our in vivo (beads) result was in contradiction with another study from James Wells (unpublished data). In their study it was claimed that FGF4 did alter the morphology of endoderm when implanted around stage 15. We, however, found that embryos implanted with FGF4 bead at stages 9, 10.5, 13 or 15 bead showed no visible abnormality. We have demonstrated that the FGF4-bead used was active as it induced vesicle formation in animal caps, indicating that the lack of result is not due to lack of activity.

However at this time we are not able to completely discount James Wells' result as there might be technical differences that could account for

the different observations. First of all, the beads are not all the same size and there is a possibility that James Wells might have used a larger or smaller sized bead in his implant, resulting in a difference in the amount of FGF4 delivered to the embryo. Also the exact position of implantation of the FGF4-bead might be different. However, at this time, based on our own results we are inclined to say that FGF4 are not involved in the specification or development of the endoderm.

The complementary inhibitor study was found to be in agreement with the growth factor study as inhibitor treatment on endo+meso explants also resulted in normal expression of *Xlhbox8* and *Xcad2*. However we can only say for certain that noggin, SU5402 and cyclopamine are not able to repress specification of the endoderm at tne concentrations tested as we do not have a satisfactory positive control result for follistatin, sFRP-2, EGF/EFRB-2 and PD98059. Ideally one would spend the time to investigate and characterise the phenotypes of each of these inhibitors, however this is a very time consuming task as we have to try various different concentrations and ideally to confirm repression of the target biochemical pathway probably with western blots. Considering the difficulties in setting up western blots for phosphorylated proteins with *Xenopus* embryos, we decided that this was not a worthwhile effort given the limited time available.

There are several possible explanation for the lack of respecification seen in the explants. One possibility is that the specification of the endoderm might involve other growth factors not on the list. Our assay depended on using commercially available protein growth factors and our selection was limited by this. One protein that was not available commercially at the time of the experiment was *Sonic Hedgehog (Shh)*. *Shh* is a homolog of the *Drosophila* segment polarity gene *hedgehog*. It has been shown, in chick, to be involved in mediating pattern in several regions of the embryo including the limb bud (Riddle et al., 1993), somite (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994) and neural tube (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994). With regards to

endoderm development *Shh* is an interesting molecule as its earliest expression is restricted to the endoderm of anterior and caudal intestinal portals prior to invagination (Roberts et al., 1995). It has been suggested that *Shh* might be the initiating signal in the epithelio-mesenchymal interaction that takes place during endoderm specification, as in chick its receptors are found in the mesoderm and overexpression of *Shh* in the early primitive gut leads to a mesodermal and not endodermal phenotype (Roberts et al., 1998). However we must note that the mouse knockout for *Shh* has been shown to develop gut with severe foregut abnormalities (Litingtung et al., 1998; Pepicelli et al., 1998). These null mutants have malformed esophagi with enlarged lumens and disorganised or absent subjacent mesoderm.

There is also the possibility that the relevant factor(s) could be novel molecule(s). Recently Aaron Zorn has completed a microarray focussing on anterior endoderm development (unpublished data). This microarray has uncovered several novel genes that might be responsible for the patterning of the endoderm. The main technical challenge in investigating the commercially unavailable growth factors or novel genes is in producing the proteins. RNA injections would not be appropriate for investigating regional specification as it would be hard to control when the RNA is translated. RNA overexpression of growth factors might lead to disruption of the earlier stages of development. Also the injected RNA itself might decay before reaching the stage of interest.

One method that would be appropriate to produce the necessary protein is by using oocytes. Manually defolliculated oocytes when injected with mRNA coding for the protein of interest continually produce and secrete that protein when cultured in oocyte culture medium (OCM). This method has been used previously with animal caps to successfully constitute inductive signaling (Lustig and Kirschner, 1995). This test then could be adapted to study endoderm respecification by putting on the endoderm or endo+meso explants on the injected oocytes. However this method would be very

labour intensive and as a consequence might be difficult to generate a statistically significant number of results.

Multiple collaborative signals instead of a specific signal transduction pathway may also be involved in specification of the endoderm. The collaborative effect of two or more pathways has been seen in the developmental mechanism of other organs. In eye development interplay between hedgehog (HH), RA and FGFR signalling specifies the ventral regionalisation of the eye (Lupo et al., 2005). Overexpression analysis of the three signals shows that the *Xenopus* eye gets more ventralised when more than one pathway is overexpressed. Similar interactions were also seen in neural induction where three different pathways IGF, FGF and anti-BMP (chordin) induced neural fate by inhibiting Smad-1 activity (Pera et al., 2003). Smad-1 activity was shown to be inhibited at two locations. BMP antagonists such as noggin and chordin inhibits the phosphorylation of the carboxy terminal serines (De Robertis et al., 2000; Weinstein and Hemmati-Brivanlou, 1999), whilst FGF8 and IGF2 activates MAPK which in turn phosphorylate the Smad-1 linker region further inhibiting its activity (Kretzschmar et al., 1997; Pera et al., 2003). These two actions act together to downregulate Smad-1 activity and thus promote neurogenesis. Looking at these examples of synergy between different pathways raises the possibility that specification of the endoderm might involve more than one pathway and simultaneous activation might be necessary to induce specification. To address the possible necessity for multiple pathways to be activated in endoderm specification, the growth factor screen would have to be performed using pools of growth factors instead of just one growth factor per treatment.

In conclusion, the growth factor and inhibitor screen performed here have shown that none of the molecules listed in Table 4.1 is sufficient on its own to induce regional specification of the endoderm. In future the screen could be expanded either to test for multiple signals for specification or using oocytes to test novel or commercially unavailable gene products.

V. Heterologous Recombinations of Mesoderm and Endoderm

V.1. Introduction

Heterologous recombination experiments have provided great insights into the interactions between mesoderm and endoderm in the regional specification of the endoderm. By recombining endoderm with mesoderm from different regions one can start to understand how the endoderm is specified along its A-P axis. The earliest homologous recombination experiment with the endoderm was done in the newt, *Cynops* (formerly *Triturus*) *pyrrhogaster*. Here it was demonstrated that a significant amount of mesoderm is required for the proper specification of the endoderm (Okada, 1954a; Okada, 1954b; Okada, 1955a; Okada, 1955b). Further recombinations with the newt showed that the mesoderm has an instructive influence on the endoderm. Endoderm that would normally develop to form pharynx can be respecified to produce more posterior organs when recombined with lateral mesoderm. Similarly anterior and middle endoderm explants can also be respecified to form pharynx when put near head-mesenchyme (Okada, 1955a; Okada, 1955b; Okada, 1957; Okada, 1960). However these recombinations were done with relatively later stages, indicating that this is testing more for the plasticity of the late tissues rather than the initial specification event.

Also these studies have several important technical limitations. At the time, due to a lack of a specific lineage tracers, no detailed fate map was available. This meant that comparisons could not be made between experimental results and the presumptive fate for that particular piece of tissue. There was also a lack of molecular markers that would label the different parts of the developing gut. As a consequence the different parts of the gut were identified histologically, a much less reliable method compared to using molecular markers.

In mouse, heterologous recombination using tissues from forestomach and glandular stomach at later stages showed no respecification. Here it was shown that forestomach epithelium could not be respecified when recombined with glandular stomach mesenchyme. The opposite recombination also showed that the glandular stomach epithelium maintains its differential fate when recombined with forestomach mesenchyme (Fukamachi et al., 1979). Similar observations were seen when the experiment was performed in rats (Fukamachi and Takayama, 1980). However these recombinations were done with tissues from late embryos (14.5 – 18.5 day embryos) and as such the endoderm may have already become determined and or the mesoderm lost its signalling specificity, accounting for the lack of respecification seen.

In chick, heterologous recombinations were also used to help understand how endoderm and mesoderm interact in development. Gizzard (muscular stomach) in chick would normally be devoid of any glands at hatching. However when gizzard endoderm is recombined with proventricular or intestinal mesenchyme it goes on to develop proventriculus-type and intestinal-type endocrine cells respectively (Andrew and Rawdon, 1990; Andrew et al., 1988; Rawdon and Andrew, 1988). Recently a more detailed analysis of chick endoderm respecification on 10-somite stage embryos showed a posterior-dominant relationship between mesoderm and endoderm in chick (Kumar et al., 2003). Here the endoderm was shown to only be respecified when recombined with a more posterior mesoderm. For example *Pdx1*, normally expressed in midgut could be induced when recombined with midgut lateral plate mesoderm (LPM). Also induction of *CdxA* and inhibition of *Pdx1* was seen in midgut endoderm when recombined with hindgut LPM. Recombinations with more anterior LPMs only resulted in the maintenance of the current endodermal anteroposterior fate (Kumar et al., 2003).

In *Xenopus*, heterologous recombinations between anterior and posterior endoderm and mesoderm from stage 20 explants showed that the

mesoderm patterns the endoderm in an instructive manner (Horb and Slack, 2001). In this study it was shown that *Xlhbox8* expression was suppressed when anterior endoderm was recombined with posterior mesoderm. On the other hand *Xlhbox8* expression was induced when posterior endoderm was recombined with anterior mesoderm. However in this study the change in expression of markers was only demonstrated using PCR. Since our growth factor screen used *in situ* instead of PCR, we thought it would be essential to complete the heterologous recombination data with *in situ*. This would help demonstrate that the detection method we chose for the screen was sufficiently sensitive to detect any induction or respecification that might be caused by growth factor treatment. It would also help to confirm that the lack of respecification observed in the growth factor screen was because the factors tested are not involved in endoderm specification.

V.2. Materials and Methods

To generate the heterologous recombinants, endoderm and mesoderm were cut and divided into anterior and posterior halves (see Fig 5.2 A for diagram). These cuts were done in NAM/2 in the presence of trypsin to allow complete separation of endoderm and mesoderm. Anterior endoderm and posterior mesoderm was transferred to NAM/2 solution with anti-trypsin, and recombined. The recombination was done in a small well in the agar made with forceps. A piece of glass was put on top of the recombinant to keep the tissues in place. They were then allowed to heal for approximately 1 hour before being transferred to fresh NAM/2 + 1%BSA for culture. Posterior endoderm and anterior mesoderm recombinants were also made similarly.

For the control halves, the endoderms were cut out from the embryo with the mesoderm and cut in half in NAM/2 solution without trypsin according to the diagram in Fig 5.2 A. They were then allowed to heal and transferred to fresh NAM/2 + 1%BSA for culture. All the explants were cultured until the control embryos reached stage 42 before being fixed for in situ with *Xlhbox8, Xcad2 and Edd*

V.3. Results

V.3.1. Stage 20 heterologous recombinations

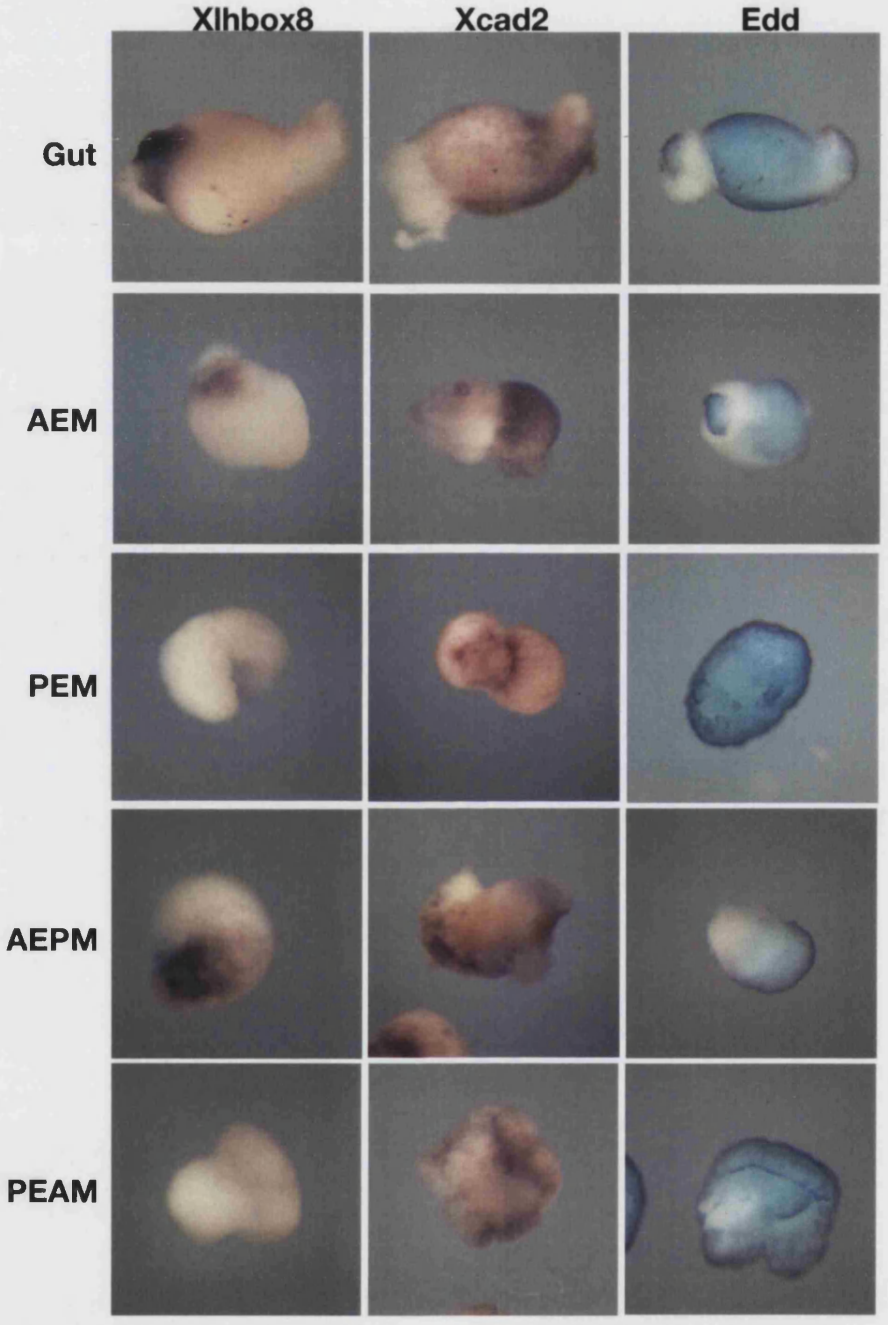


Figure 5.1 In situ on stage 20 heterologous recombinations.
The pictures above show in situ hybridisation done on anterior (AEM) and posterior (PEM) half explants as well as the heterologous recombinations between anterior endoderm and posterior mesoderm (AEPM) and posterior endoderm and anterior mesoderm (PEAM). Isolated stage 42 gut here acts as a positive control for the makers. The expression of the markers *Xlhbox8* in anterior endoderm explants and *Xcad2* in all explants indicate that mesoderm is not respecifying endoderm in these recombinations.

The results from the *in situ* were not what we expected (Fig 5.1). Here our heterologous recombinations seemed to show that the mesoderm was only playing a permissive role and not an instructive one as previously found (Horb and Slack, 2001). Anterior endoderm still shows expression of *Xlhbox8* when recombined with posterior mesoderm. Similarly posterior endoderm shows no expression of *Xlhbox8* or downregulation of *Xcad2* when recombined with anterior mesoderm. This pattern is similar to that of the control AEM and PEM explants. To confirm that this lack of respecification of the endoderm was not because of the detection method used (*in situ* and not PCR), we decided to repeat the experiment using PCR to detect expression of *Xlhbox8, Xcad2* and *EF1-α* (Fig 5.2 A).

Figure 5.2 PCR of stage 20 heterologous recombinations.
A). Diagram on the left shows how the stage 20 endoderm was halved. The PCR was done for the markers *Xlhbox8*, *Xcad2* and *EF1-α* in the half explants. Results here confirmed that seen with the in situ as *Xlhbox8* was expressed with anterior endoderm regardless of whether it remains with anterior or with posterior mesoderm. Whilst posterior endoderm only expresses *Xcad2*. B) The diagram on the left shows how the endoderm was divided into 4 regions: extreme anterior (EA), anterior (A), posterior (P) and extreme posterior (EP). Explants done with quarters showed recombination of the extreme anterior and extreme posterior pieces. Extreme anterior endoderm (EAE) surprisingly lost expression of *Xcad2* when recombined with extreme posterior mesoderm (EPM). Extreme posterior endoderm (EPE) did not show expression of *Xlhbox8* when recombined with extreme anterior mesoderm (EAE). All recombinations was performed at least in triplicate.

The PCR results confirmed the *in situ* observations. Anterior endoderm maintains expression of *Xlhbox8* when recombined with posterior mesoderm and posterior endoderm shows no expression of *Xlhbox8* when recombined with anterior mesoderm. At this point it looked very likely that Horb and Slack's result was not repeatable. However there is still the possibility that the anterior and posterior halves of the embryo was not separated enough. Because the halves were made arbitrarily there is a possibility that there might be some overlap of tissue between the two halves. This overlap of tissue might result in a posterior signal being present in anterior mesoderm inhibiting expression of *Xlhbox8* in posterior endoderm. Similarly, anterior signal might be present in the posterior mesoderm maintaining *Xlhbox8* in anterior endoderm.

To address this we decided to do a recombination based on quarter embryos. The extreme anterior and extreme posterior regions are on opposite ends of the endoderm, which means that there should be no overlap in explants made from these regions (Fig 5.2 B). Results show that control extreme anterior endo+meso explant (EAEM) expressed both *Xlhbox8* and *Xcad2* whilst extreme posterior endo+meso explant (EPEM) only expressed *Xcad2*.

Extreme posterior endoderm maintained its expression of *Xcad2* when recombined with extreme anterior mesoderm indicating no respecification occurred. However when extreme anterior endoderm was recombined with extreme posterior mesoderm *Xcad2* expression was lost whilst *Xlhbox8* was maintained. This was unexpected as posteriorisation of the anterior endoderm should lead to the suppression of *Xlhbox8* and not *Xcad2*.

Initially we suspected that this might be an artefact as a result of the recombination procedure (e.g. the mechanical compression or tissue mass augmentation). To test for this we performed homologous recombinations with the extreme anterior and extreme posterior quarters. Extreme anterior endoderm and mesoderm were separated in NAM/2 solution with trypsin,

moved to NAM/2 + trypsin inhibitor, recombined and helped into place with a piece of glass. The recombinants were then allowed to heal for an hour before being transferred to fresh NAM/2 + 1%BSA for culture. Homologous extreme posterior recombinants was also made in this way. RT-PCR for *Xlhbox8, Xcad2* and *EF1-α* on these explants is shown in Figure 5.3.

Here we see that the extreme anterior quarter homologous recombinant still maintains expression of both *Xlhbox8* and *Xcad2* unlike the heterologous recombinant between extreme anterior endoderm and extreme posterior mesoderm. This then confirms that the downregulation of *Xcad2* in these explants was real and not an artifact. We can then conclude from this that the extreme posterior mesoderm does respecifiy the extreme anterior endoderm albeit not in the manner expected.

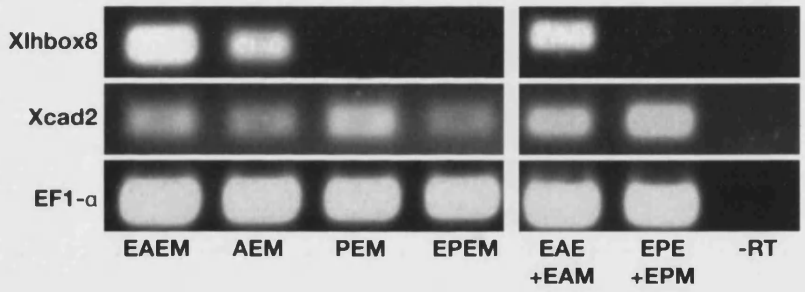


Figure 5.3 Homologous recombination.
The panels on the right show PCR results for *Xlhbox8*, *Xcad2* and *EF1-α* for the homologous recombinations with the extreme anterior and extreme posterior quarters. Here we see that Xcad2 expression is maintained in extreme anterior homologous recombination. Results from the control quarter explants are shown on the left.

V.3.2. Stage 20 Mesoderm and early Endoderm recombinations

We had established that the result on heterologous recombination obtained by Horb and Slack was not reproducible. The possible reasons for these differences are discussed at the end of this chapter. However considering that we performed at least three *in situs* and four repeats of the RT-PCR in the heterologous recombination, we are inclined to believe that our observation is the correct one: mesoderm from stage 20 mesoderm was not able to direct the specification of stage 20 endoderm.

One interpretation of this lack of respecification is that the mesoderm explants from stage 20 were only playing a maintenance role and not an instructive role. If the mesoderm was only maintaining a previously existing pattern, there is a possibility that the endoderm acquired its patterning at an earlier stage of development, prior to stage 20, but that it does not become stable until a later stage. Based on this we decided to do more heterologous recombinations using endoderm from earlier stage embryos (stage 10.5 and 13). We decided not to use stage 9 because of the previously reported phenomenon of appearance of mesoderm in endoderm explants (whether by contamination or regulation) making the pregastrulation vegetal explant unreliable for this type of study. Whole endoderm from these early embryos was isolated and then recombined with stage 20 mesoderm (anterior or posterior). We chose not to use earlier mesoderm in the recombinations as it was not possible to generate separate anterior and posterior mesoderm explants from early stage embryos making it unsuitable for studying respecification.

Whole endoderms from stage 10.5 and 13 embryos were isolated as shown in the top of Figure 5.4. The cut from stage 10.5 avoids the invaginating edge of the endoderm as there have been evidence that this leading edge of the endoderm already expressed *XHex* and *Cerberus*, markers of anterior endoderm development (Zorn et al., 1999). This indicates that this leading edge might already received patterning at stage 10.5. Thus, inclusion of this leading edge in the explant might lead to possible complications in interpreting the results.

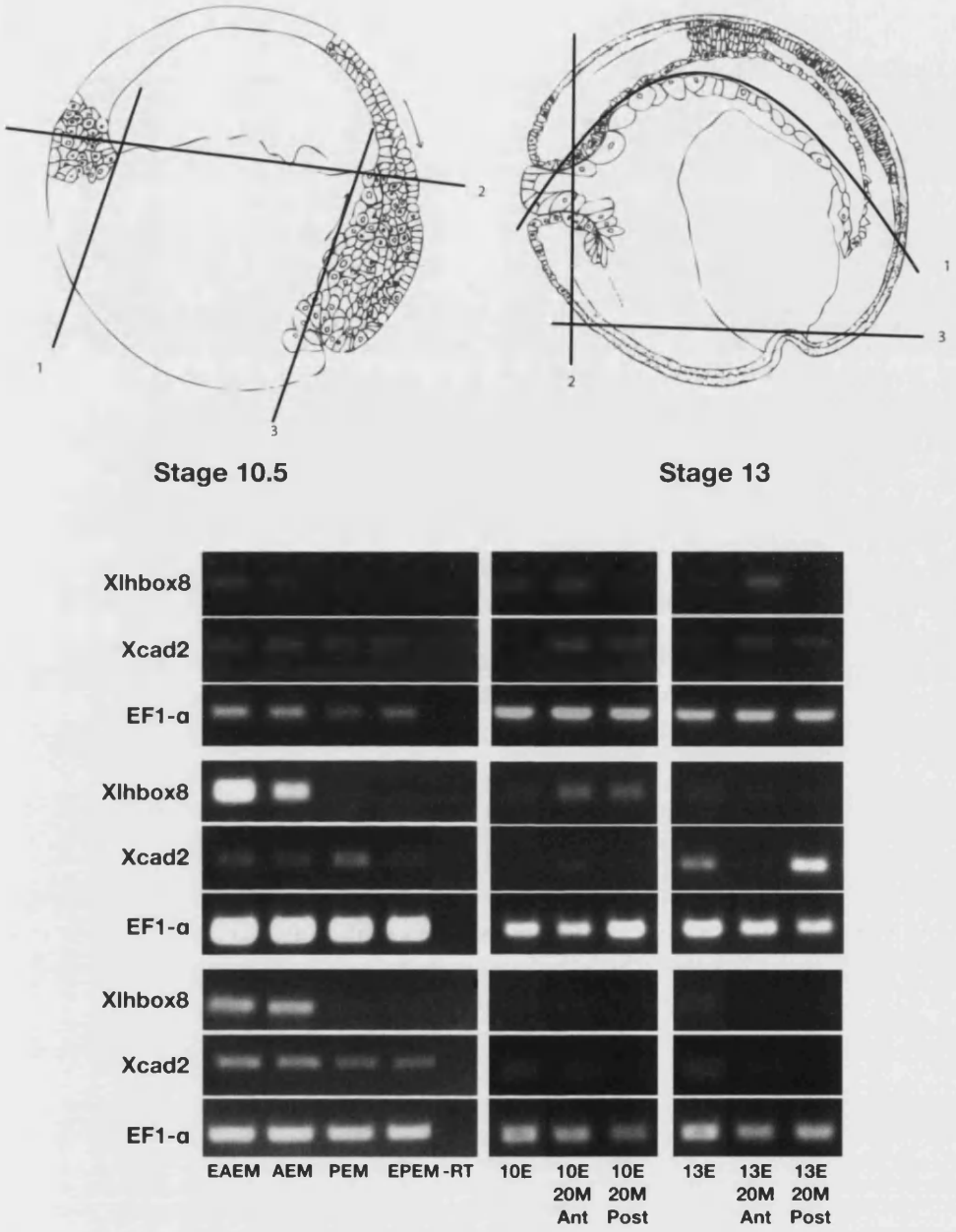


Figure 5.4 Stage 10.5&13 recombinations.
The diagram on the top shows how the endoderm from stage 10.5 and 13 embryos was isolated. They were then recombined with either anterior or posterior stage 20 mesoderm. 15-20 of each explants were then collected and tested for the markers *Xlhbox8*, *Xcad2* and *EF1-α*. From the three PCR reactions shown above we saw that eventhough the expression pattern of the controls based on the quartered explants were constant the recombination and endoderm from stage 10.5 and 13 embryos vary from reaction to reaction.

With stage 13 embryos, the cut was made so that the thin flap of the endoderm that was attached to the roof of the archenteron was preserved in the explant. This was done by making the initial dissection from opposite the yolk plug and teasing gently the thin endoderm until it was detached from the roof of the archenteron. Once the endoderm was isolated it was recombined with either anterior or posterior mesoderm halves from stage 20 embryos. The recombinant was allowed to heal in NAM/2 solution under a piece of glass for 1 hour. Once healed the recombinant was then transferred to fresh NAM/2 + 1%BSA for culture. Once the control embryos reached stage 42 the explants were collected for RT-PCR with *Xlhbox8*, *Xcad2* and *EF1-α* (Figure 5.4).

The PCR for these early recombinants were found to be unreproducible. Figure 5.4 shows PCR results for 3 different recombinations with stage 10.5 and 13 endoderm as well as the control explants. Analysis on the first recombination (the upper right panel) showed a result similar to the Horb and Slack result with *Xlhbox8* induced by anterior mesoderm and not posterior mesoderm, suggesting regionally specific programming by the mesoderm. However, different repeats of the experiments with recombinants of stage 10.5 and 13 endoderm showed different expression of the markers. Sometimes we found *Xlhbox8* expressed in the anterior, sometimes in the posterior, sometimes in both anterior and posterior and othertimes in none. We also found similar unreproducible expression of *Xcad2* with stage 10.5 and 13 explants. Because the expression of the markers *Xlhbox8* and *Xcad2* were consistently repeatable with the control stage 20 quarter explants we concluded that this unreproducible observation with the stage 10.5 and 13 endoderm was due to the way the recombinant/explant was generated.

Initially we thought that there was not enough contact between stage 20 anterior or posterior mesoderm halves and the whole endoderm of stage 10.5 and 13 embryos. The whole endoderm is approximately 3-5 times larger than the mesoderm halves, thus in the recombinants only part of the

stage 10.5 and 13 endoderm were in contact and received signals from stage 20 mesoderm. To test for this we decided to recombine 3 stage 20 anterior or posterior mesoderm halves with one endoderm from stage 10.5 or 13. This would help ensure that most of the endoderm was in contact with the mesoderm. However when we analysed these explants we saw the same unreproducible expression of *Xlhbox8* and *Xcad2*. This then meant that the unreproducible pattern of expression was not due to a lack of contact between the endoderm and mesoderm in the recombinant.

Another possible source for the unreproducible expression pattern might be contamination. We identified two possible sources of contamination in making the recombinant. First, there could be endoderm attached to the isolated mesoderm from stage 20 which could contribute to the expression of *Xlhbox8* and *Xcad2* in the recombinant. Second, there could be contaminating mesoderm in the endoderm explants.

To address the first possibility we decided to isolate stage 20 anterior and posterior mesoderm halves and analyse them for expression of *Xlhbox8, Xcad2* and *EF1-α*. This will allow us to see if they contributed to the expression of *Xlhbox8* and *Xcad2* seen in the recombinations. Results of the RT-PCR halves showed that the isolated stage 20 anterior and posterior mesoderm had no expression of *Xlhbox8* or *Xcad2* (Figure 5.5A). Thus indicating that the stage 20 mesoderm used in the recombinations did not contribute to the unrepeatable results observed earlier.

To address the second possibility of whether or not there were mesodermal contaminations in the endoderm explant from stage 10.5 and 13 embryos, we decided to test for the expression of the mesodermal markers xFOG and FoxF1. The mesodermal markers *xFOG* and *FoxF1* are normally expressed in the mesoderm around the endoderm and have been previously used in detecting mesodermal contaminations in endoderm explants (Deconinck et al., 2000; Horb and Slack, 2001; Koster et al., 1999). We isolated stage 20 endoderm and endo+meso explants as well as whole endoderm from stage 9, 10.5 and 13 to test with these markers. Stage 20

anterior and posterior mesoderm were also collected as positive controls. The recombinants were not tested as they contained stage 20 mesoderm. We also decided not to test more than these two markers as expression of these two markers would be enough to determine whether or not there was mesodermal contamination and that additional markers would not provide any additional information.

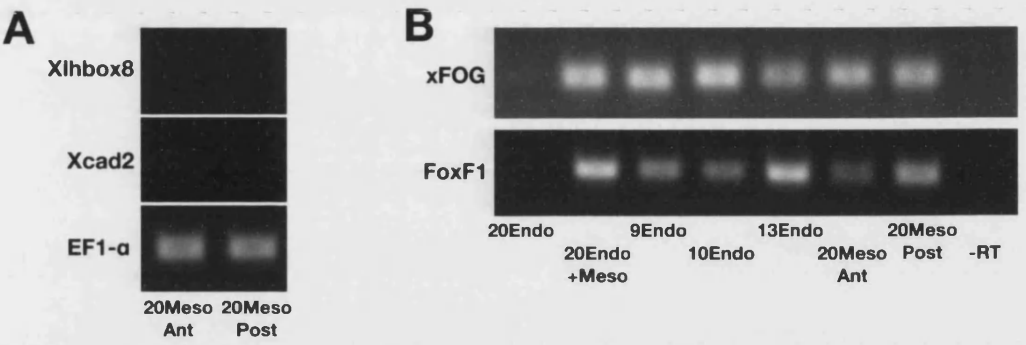


Figure 5.5 Testing for contaminations.
A). Shows PCR for Xlhbox8, Xcad2 and EF1-α done on 20 mesoderm only explants taken from stage 20 embryos. B). PCR for the mesodermal markers xFOG and FoxF1 done using various different explants to see if mesoderm might be present in the endoderm only explants.

The RT-PCR with the mesodermal markers *xFOG* and *FoxF1* revealed that the whole endoderm from stage 10.5 and 13 embryo did contain a significant amount of mesodermal contamination (Figure 5.5B). This then suggests that the unreproducible expression pattern of *Xlhbox8* and *Xcad2* observed earlier may be due to mesodermal contaminations in the whole endoderm explants. We were also able to confirm previous observations that vegetal explant from stage 9 embryo contained mesodermal tissue and that stage 20 endoderm explants can be made to be free of mesoderm (Horb and Slack, 2001).

V.4. Discussion

One of the essential results of the paper of Horb and Slack (2001), was that the endoderm only becomes specified after stage 25. This result has been amply confirmed in our present study as *Xlhbox8* and *Xcad2* expression is only maintained when the endoderm from stage 20 embryo is

cultured with mesoderm. However, our *in situ* and PCR results with the half explants here demonstrated that the mesoderm does not pattern the endoderm in an instructive manner as was previously reported in (Horb and Slack, 2001). We did not observe any induction of *Xlhbox8* expression in posterior endoderm when recombined with anterior mesoderm and similarly *Xlhbox8* expression was not downregulated in anterior endoderm when recombined with posterior mesoderm. Instead it seems from the current results that any mesoderm (anterior or posterior) will provoke a re-activation or a maintenance of *Xlhbox8* in the anterior and *Xcad2* in the middle and posterior of the endoderm. This then indicates that the mesoderm might only be acting in a permissive role at these stages and not instructing endoderm development as previously thought.

There was one notable exception to the lack of respecification in the heterologous recombinations. This was seen with the quarter explants in which *Xcad2* expression is lost when the anterior most (EA) endoderm is recombined with the posterior most (EP) mesoderm (See Figure 5.2B for details of segment in quarter explants). This was extremely surprising considering *Xcad2* is a posterior marker and one would expect it to be induced instead of downregulated by EP mesoderm. We have confirmed that this loss of *Xcad2* expression is reproducible and was not an artifact of the recombination itself as *Xcad2* expression is maintained when EA endoderm is separated and then recombined with another EA mesoderm.

At the moment we do not have a clear idea as to the possible mechanism that would account for this particular patterning event. Our closest model involves a signalling centre that is positioned in the middle of the embryo. Whilst this would account for the loss of *Xcad2* when EA endoderm is recombined with EP mesoderm, it does not account for the maintenance of *Xcad2* in EP endoderm and EP mesoderm homologous recombination. To be more certain of what exactly is going on more heterologous recombinations need to be made between the EA endoderm and mesoderm from the other types of quarter explant (A and P see Fig 5.2

B). If the downregulation of the *Xcad2* expression is isolated to the EA endoderm and EP mesoderm recombination then there might be a positional mechanism involved. On the other hand if the downregulation is observed in all the recombinations then it might indicate that the expression of *Xcad2* in the EA endoderm is very labile and can only be maintained by the presence of EA mesoderm.

Even though there was an anomaly with the quarter explant heterologous recombinations, our results with the half explant heterologous recombinations are clearly different from that presented in Horb and Slack (2001). There are several possible explanations for the different results which we will consider. First there is the possibility that the explants cut by Marko Horb from stage 20 endoderm differ slightly from the ones generated in this study. The anatomical markers for where the cuts were made in generating the explants are somewhat arbitrary. For example, Marko might have cut slightly more dorsal to separate the explant from the embryo or that he might have cut more anterior or posterior than we have resulting in a longer explant along the A-P axis. It is then conceivable that the extra tissue could be expressing the instructive signals which would in turn respecify the endoderm. We also need to consider the opposite situation, Marko Horb might have cut slightly less ventral or anterior and posterior resulting in a smaller shorter explant. The smaller piece of tissue then might have lost a source of inhibitory signal that would result in the signals sent by the mesoderm becoming instructive instead of permissive.

In this interpretation, two separate signalling centres must exist in the mesoderm that patterns the endoderm. When only one is present the mesoderm would behave in a permissive manner and maintain expression of the specification markers in the endoderm. On the other hand when both signalling centres are present and active the mesoderm would behave in an instructive manner and be able to induce respecification in the endoderm. We have considered previously, in Chapter 4, cooperation between different signalling pathways and how they have been shown to exist in the

development of the eye and the neural plate (Lupo et al., 2005; Pera et al., 2003). If this hypothesis were true then screening the endoderm explants using a multiple signal approach, as was discussed in Chapter 4 might reveal the existence of cooperative signals that are instructive when together and permissive on their own.

Another possible explanation for the difference between our results and Marko's is the wide genetic variability in *Xenopus*. Most of the *Xenopus* used in science is still supplied from the wild. Frogs with different genetic backgrounds might behave slightly differently to treatment. We recently discovered this with the refractory period of regenerating tail. Normally a cut tail in *Xenopus* would be regenerated. However there is a period of development where the cut tail does not regenerate, this is called the refractory period and lies between stage 45-47 (Beck et al., 2003). This was characterised using ten spawnings from the wild caught South African frogs (from *Xenopus* Express) in use in the lab at the time. Since then, problems with disease prompted a switch to lab-reared frogs from NASCO. With tadpole batches from the NASCO frogs there is often no refractory period. We need to note however that the refractory period, when it occurs, does so late in development and involves tissue that would be lost during metamorphosis.

We do not expect the same level of variation to exist for the development of the endoderm as it occurs much earlier and involves tissues that are essential for the entire lifespan of the animal. Indeed throughout this entire study we have gone through 4 different batches of *Xenopus laevis* from three different suppliers (Blades, *Xenopus* Express and NASCO) making it unlikely that the source of the difference in results is from the source of the *Xenopus* used.

Lastly we need to consider the possibility that Marko Horb might have made not made enough repetitions in his observation (Horb and Slack, 2001). As we discussed previously, the position of the cuts in the experiment was arbitrary. This then increases the possible variability of the

results. It is possible that in his studies Horb did not repeat it often enough and as a result he only observed the apparent instructive role of the endoderm. Also his experiments showed RT-PCR results from the heterologous recombinations and not *in situ* hybridisations data. Perhaps if he had performed the in situ, it would bring attention to the possible variability of this method. In our own repetitions we found that all of them showed a permissive mesoderm.

Thus, assuming that our result is the correct result, it is then possible that the endoderm might be patterned at an earlier stage and that the subsequent role of the mesoderm is entirely permissive. We have repeatedly observed through both PCR and *in situ* hybridisation that at stage 20 the mesoderm was not able to respecify the endoderm. Instead, it was only capable of re-activating or maintaining normal expression patterns of *Xlhbox8* and *Xcad2* in the endoderm. Thus, it follows that if the specification markers can only be re-activated or maintained by the mesoderm at stage 20 then there must be another patterning event that occurred earlier to give the endoderm its identity along the A-P axis. We attempted to identify possible stages when this event might have occurred by making endoderm-only explants from earlier stages (stage 10.5 & 13). However we later found that explants made from these stages to be contaminated with a significant amount of mesoderm resulting in variable expression patterns of *Xlhbox8* and *Xcad2* with heterologous recombinations involving endoderm from stage 10.5 and 13 embryos.

The existence of a significant amount of mesoderm in the explants meant that this method is unsuitable for studying endoderm respecification in early embryos. We need to consider other methods that would affect the endoderm development *in vivo*, avoiding the mesodermal contamination altogether. The transgenic method is a very powerful and flexible tool for overexpression of genes in *Xenopus*. In order to adapt it to study endoderm we need to have spatial and temporal control, so that the transgenes are only overexpressed in the mesoderm at the stage of interest.

Our lab has previously created a transgenic construct containing the heat shock promoter Hsp70 (Beck et al., 2003). Under the control of this promoter, genes are only expressed when the embryos are subjected to a temperature of 34°C, thus allowing for temporal control of the expression of the gene of interest. To gain spatial control for the expression we need to use another well known method in *Xenopus*, grafting. Microsurgery is relatively easy in *Xenopus* embryos since the embryos are relatively large and heal well after surgery. Previous work in our lab has shown that it is possible to make specific grafts of presomitic mesoderm from stage 14/15 embryo (Gargioli and Slack, 2004). Thus to restrict the expression of the growth factors specifically to the mesoderm one can graft the mesoderm from that particular stage of development from a heat shocked transgenic embryo to a wildtype recipient.

The discovery that the result from heterologous recombinations on stage 20 embryos (Horb and Slack, 2001) were not repeatable has certainly raised a lot of new questions regarding the timing and mechanism of regional specification of the *Xenopus* endoderm. From the various hypotheses discussed here we feel that the most probable one is that at stage 20 the mesoderm is only acting in a permissive role, maintaining an earlier, labile, patterning event. The next step is then to investigate the precise timing when this patterning event takes place. We have shown that this investigation could not be carried out using the explant system as it is difficult to remove completely the mesoderm contamination. This also means that we cannot use heterologous recombination to try and identify at which stage the patterning of the endoderm actually occurs. This then makes identifying the early patterning event much more difficult as we must rely on misexpression or overexpression of various growth factors at various stages to hopefully identify exactly when the earliest endoderm patterning event occurred. Both the transgenic and the implanted bead approach may be applicable to solving this problem.

VI. *Hox* genes and the endoderm

VI.1. Introduction

Data from heterologous recombinations of endoderm and mesoderm in chick, mouse and *Xenopus* have shown that the mesoderm plays a pivotal role in specifying the endoderm along the A-P axis (Dessimoz et al., 2006; Horb and Slack, 2001; Kumar et al., 2003; Wells and Melton, 2000). The mesoderm is thought to send out different signals to pattern different parts of the endoderm depending on its position along A-P axis. In order to do this the mesoderm must be able to tell its own positional identity along the A-P axis. It has been suggested that the *Hox* genes might be involved in providing this positional identity in the mesoderm.

Hox genes are a subset of the *homeobox* genes which encode transcription factors containing the 60-amino acid homeodomain (McGinnis and Krumlauf, 1992). They are excellent candidates for providing positional information along the A-P axis of the endoderm as they have been shown to play an important role in the A-P patterning of the mesoderm. Mutations to the *Hox* genes have been shown to result in morphological defects that are restricted to discrete segmental zones along the A-P axis in a wide variety of animals, ranging from nematodes to mice (Pearson et al., 2005). *Hox* genes have also been shown to provide positional information along the A-P axis in the development of both mesoderm and ectoderm. In the mesoderm, the shape of a vertebra is controlled by *Hox* code, and in the hindbrain (ectoderm), appropriate neuronal differentiation has been shown to be *Hox*-dependent (Krumlauf, 1994; McGinnis and Krumlauf, 1992).

Hox genes are expressed in the correct germ layer to provide positional information in the mesoderm, as most of the *Hox* genes are found to be expressed in the mesoderm and not the endoderm. In *Drosophila* only labial is found in the endoderm (Bienz, 1997). In chick as well, most of the *Hox* genes are found to be expressed in the mesoderm whilst only a subset

are expressed in the endoderm at levels detectable by *in situ* hybridisation (Grapin-Botton, 2005).

In chick the *Hox* genes have been found to be expressed in a nested, overlapping pattern in the developing gut mesoderm (Roberts et al., 1995; Sakiyama et al., 2000; Sakiyama et al., 2001; Yokouchi et al., 1995). Furthermore, the boundaries of expression for some of these mesodermal *Hox* genes align with the morphological borders of the different gut regions further supporting their possible role in providing positional information in the mesoderm (Grapin-Botton, 2005). This is especially true for the 5' members of the *Hoxa* and *Hoxd* clusters (paralogues 9-13) (Roberts et al., 1995). The expression pattern of these *Hox* genes have been shown to match the morphological borders of the different gut regions of the posterior midgut and hindgut.

This alignment of the borders of mesodermal *Hox* gene expression and morphological borders is more significant when we consider that the *Hox* genes are master transcriptional regulators, capable of regulating the expression of many downstream genes. Through this mechanism it is then conceivable that the *Hox* genes are activating the expression of various signal molecules in the mesoderm depending on their position along the A-P axis. These different signals might then induce different fates in the endoderm along the A-P axis, resulting in the patterning of the endoderm.

In *Xenopus* similar regional expression pattern is also observed for a number of *Abdominal B*-type *Hox* genes (Lombardo and Slack, 2001). Even though the boundaries of these *Hox* genes have not been matched with morphological boundaries in the gut in *Xenopus*, it certainly raises the possibility that they might provide the mesoderm with positional information along the A-P axis. So far the *in situ*s have only been performed on whole embryos or on isolated gut, so we do not know whether the genes are expressed in endoderm or mesoderm or both layers. Thus to further build up evidence for the role of *Hox* genes in endoderm specification we aimed to investigate the expression patterns of these *Hox* genes in more detail.

As was shown previously the endo+meso explants contains the minimum amount of tissue necessary for the specification of the endoderm as they maintain expression of *Xlhbox8* and *Xcad2* (Horb and Slack, 2001). Expression of the *Hox* genes in these explants would give further support to the argument that *Hox* genes are playing a role in the specification of the endoderm. We aimed to investigate this by performing *in situ*s on endo+meso explants for the genes *xHoxA9, xHoxD9, xHoxD10, xHoxC12 and xHoxA13* which have previously been studied in whole embryo and isolated gut in *Xenopus* (Lombardo and Slack, 2001). We also included another posterior *Abdominal B*-type *Hox*, *xHoxD13* (Christen et al., 2003).

We also aimed, through this study to determine conclusively whether the *Abdominal B*-type *Hox* genes are expressed in the endoderm or the mesoderm. Previously Aurora Lombardo in her experiments showed that the *xHoxA13* is expressed in both endoderm and mesoderm. However this was done by manually hemisecting a stage 42 gut after wholemount *in situ*. We feel that this method does not have enough resolution to say conclusively whether it is in both layers or just in the mesoderm. Also the location of the other *Hox* gene transcripts have not been established. We then aimed to address this by performing sections on the gut following wholemount *in situ* hybridisation. This should provide us with the resolution needed to draw firm conclusions on whether the *Hox* genes are expressed in the endoderm or mesoderm or in both layers.

VI.2. Materials and Methods

The probes for the *in situ* were made by linearising plasmids with the appropriate enzyme and transcribing the DIG-RNA probe using the appropriate polymerase. The list of enzymes and polymerase used to make the *Hox* probes are listed in Table 6.1. It is worth noting that the development time for histochemical reaction were especially long for these *Hox* probes, up to 48 hours. This was due to the relatively low level of expression of the *Hox* genes in normal embryo. The embryos were bleached after colour development to better visualise the expression pattern.

Table 6.1 Restriction enzyme and polymerase for *Hox* probes

Target Gene	Restriction enzyme	RNA Polymerase
xHoxA9	NcoI	SP6
xHoxD9	NcoI	SP6
xHoxD10	NcoI	SP6
xHoxC12	BamHI	SP6
xHoxA13	NotI	T7
xHoxD13	EcoRI	T7

VI.3. Results

VI.3.1. Testing *in situ* probes

Before we proceeded with *in situ* on the explants, it was necessary to test the probes with stage 35 embryos to confirm that they were functional.

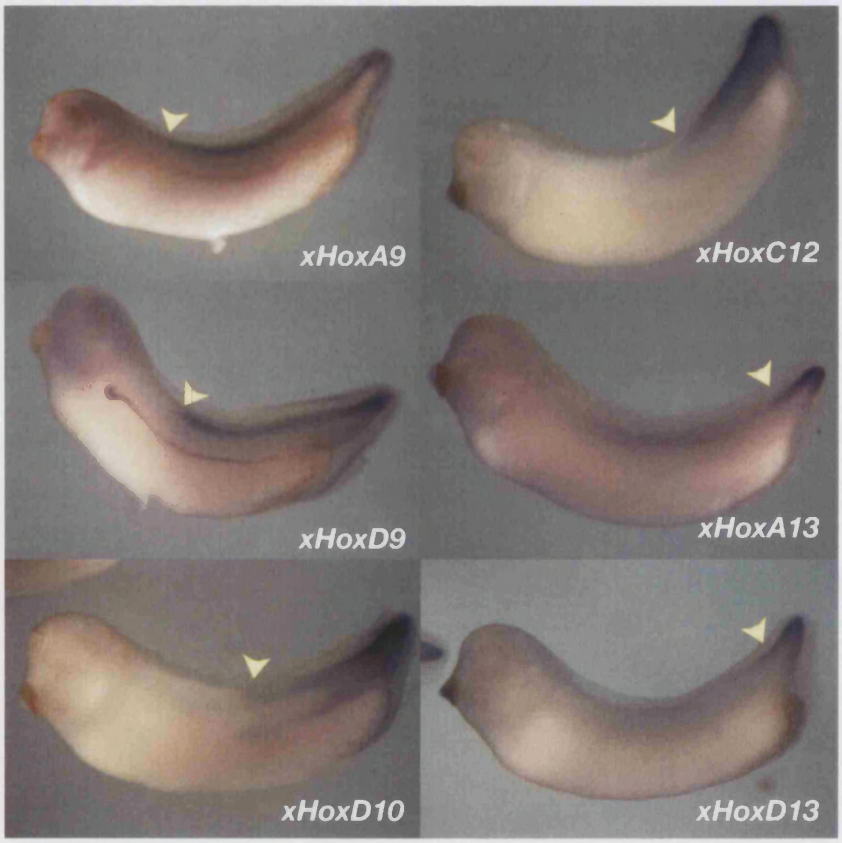


Figure 6.1 *Hox in situ* with stage 35 whole embryo.
Probes for *xHoxA9, xHoxD9, xHoxD10, xHoxC12, xHoxA13 and xHoxD13* all show the correct staining with the control stage 35 whole embryo (see Lombardo and Slack, 2001 for reference). Yellow arrow head indicate anterior boundary of expression for each of the *Hox* genes.

The results of the *in situ* hybridisations (Fig 6.1) showed that all of the probes tested were functional. *xHoxA9, xHoxD9, xHoxD10, xHoxC12* and *xHoxA13* gave patterns that have been previously described (Lombardo and Slack, 2001). *xHoxD13* also showed a previously described posterior expression pattern (Christen et al., 2003). *xHoxA9* was found to have the most anterior boundary of expression followed by *xHoxD9, xHoxD10, xHoxC12* with *xHoxA13* and *xHoxD13* having the most posterior expression.

VI.3.2. *In situ* with endoderm, endo+meso explants and stage 42 gut

Once we confirmed that the DIG-RNA probes were functional, we went on to test them with the endoderm and endo+meso explants. These explants were made as described in the Materials and Methods (Section 2.7, p45). The explants were cultured until the controls reached stage 42 before being fixed in MEMFA and processed for *in situ*. Gut isolated from stage 42 embryos were also collected to act as control and also to repeat previous experiments by Lombardo and Slack (2001).

We found that the development time was again long, approximately 36-48 hours, similar to *in situ* with stage 35 whole embryos. Results for the *in situ* are shown in Figure 6.2. Here we can see that *xHoxD9, xHoxD10 and xHoxC12* were expressed in both endo+meso explant as well as stage 42 gut but not in the endoderm-only explants. Both *xHoxD10* and *xHoxC12* showed expression in the posterior of the gut only. *xHoxD9* on the other hand showed expression in the posterior of the gut as well as a fainter stain in the anterior of the gut. These expression patterns were similar to those previously reported in *Xenopus* (Lombardo and Slack, 2001).

The staining with *xHoxA9* in the isolated gut were very faint and may simply be non-specific staining. The endo+meso staining was slightly clearer but still faint. We made three further attempts, once more with the same probe and another two times with new DIG-RNA probes, to get a clearer, cleaner staining pattern with *xHoxA9*. However we were unable to obtain a different staining for this gene in either the explants or the isolated

stage 42 gut. The stage 35 whole embryo positive control, however repeatedly showed the pattern seen in Figure 6.1. Based on these observations we were inclined to conclude that the pattern seen here is non-specific and not a real staining of *xHoxA9*.

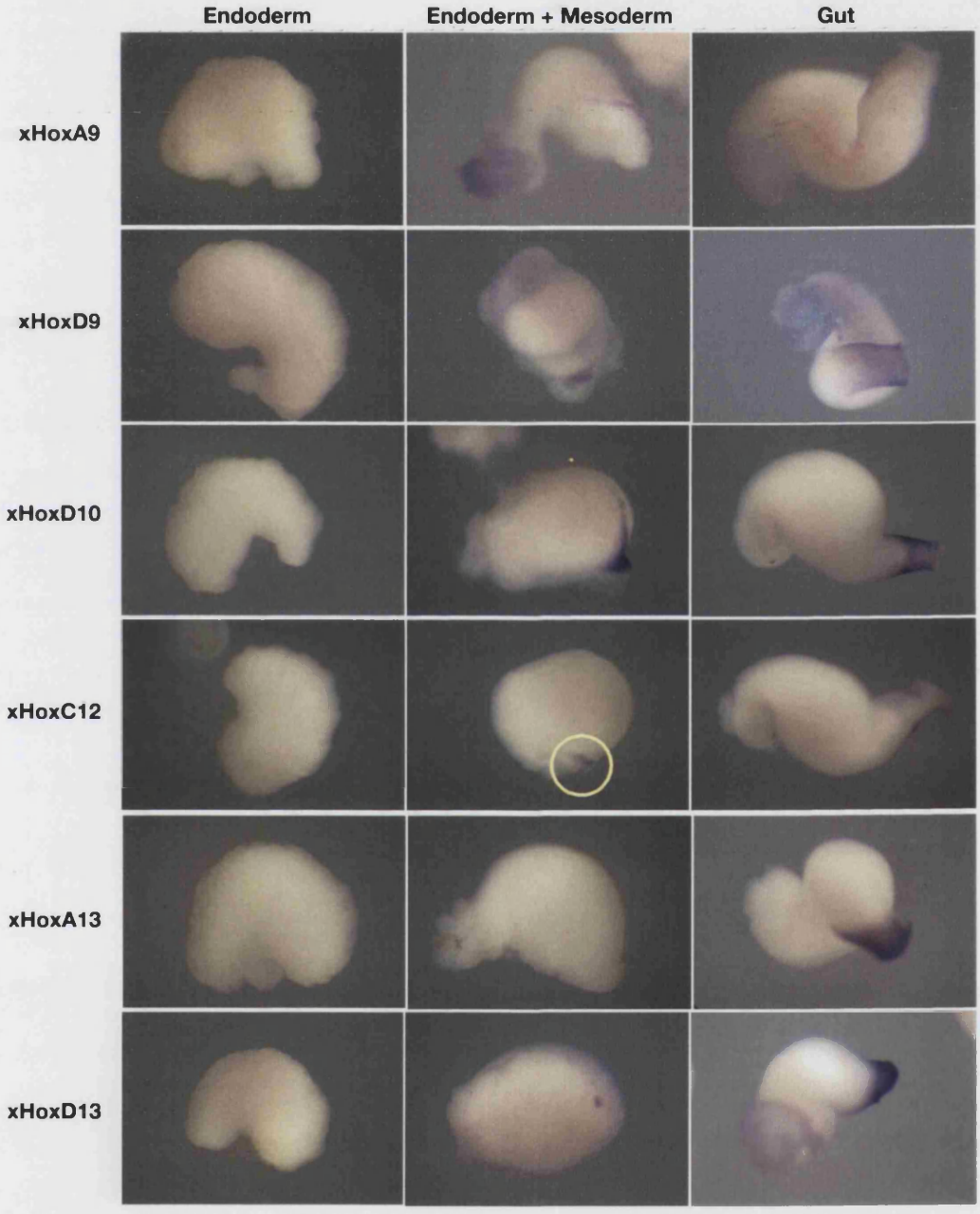


Figure 6.2 *Hox in situ.*
in situ hybridisation results with probes for the *Hox* genes *xHoxA9, xHoxD9, xHoxD10, xHoxC12, xHoxA13 and xHoxD13* done on endoderm, endo+meso explants as well as stage 42 gut. *xHoxA9* showed expression in the anterior and posterior of the gut and endo+meso explant. *xHoxD9* showed expression in anterior and posterior in the gut and endo+meso explant as well although stronger. *xHoxD10* showed a sharp band in the posterior of the endo+meso explant and a wider posterior expression domain in the gut. *xHoxC12* showed a small posterior expression domain (highlighted with yellow circle) in the endo+meso explant and a wider posterior expression in the gut. *xHoxA13* and *xHoxD13* only showed a posterior expression domain in the gut. None of the *Hox* genes tested showed expression in the endoderm only explants.

With *xHoxA13* and xHox*D13* we were only able to get a clear signal with the isolated stage 42 gut but not with any of the explants. The expression pattern of *xHoxA13* seen in the gut was similar to that previously described in *Xenopus* (Lombardo and Slack, 2001). The lack of expression of these genes in endo+meso explants were unexpected, especially considering the strong staining observed in the isolated stage 42 gut. However we need to take into account that the *xHoxA13* and *xHoxD13* were expressed at the most posterior of the embryo. Thus it is possible that the lack of staining observed with *xHoxA13* and *xHoxD13* was because the posterior part expressing *xHoxA13* and *xHoxD13* was not included in the endo+meso explant.

VI.3.3. More posterior explant expresses *xHoxA13* and *xHoxD13*

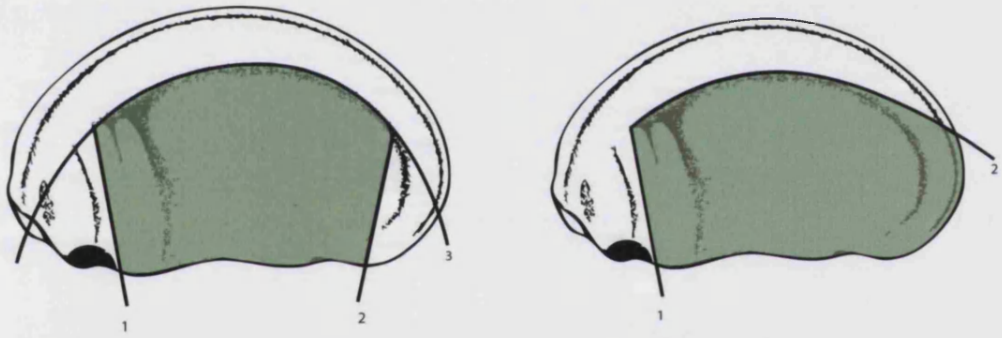
To help address the question as to whether or not the *xHoxA13* and *D13* is expressed in more posterior tissue we devised a modified cut that would generate endo+meso explants with more posterior tissue (Figure 6.3A). As can be seen from the diagram the new explant cut now contained the entire posterior of the embryo which should include the piece of tissue expressing *xHoxA13* and *xHoxD13*.

Results from this *in situ,* with the more posterior explant, show clearly that *xHoxA13* and *xHoxD13* were expressed in this new more posterior explant (Figure 6.3). This confirmed our previous hypothesis that these two *Hox* genes were expressed in the most posterior tissue not included in our original endo+meso explant.

VI.3.4. Endoderm or Mesoderm expression?

Previous *Hox in situ* in *Xenopus* have shown that at least one of the *Hox* genes, *xHoxA13* was expressed in the endoderm (Lombardo and Slack, 2001). However this conclusion was made by observing the expression pattern on gut that was hemisected after *in situ*. We felt that this was not conclusive as the resolution from this method would not be sufficient to confirm the location of the signal inside the gut. Thus to address the issue as to which germ layer each of the *Hox* genes are expressed, endoderm or mesoderm, we decided to section the wholemount *in situ* gut. The samples were fixed, processed and subsequently embedded in paraffin wax. Transverse sections were then made from these paraffin fixed samples at 10μm per section. Photographs for these sections are shown in Figure 6.4

A



B

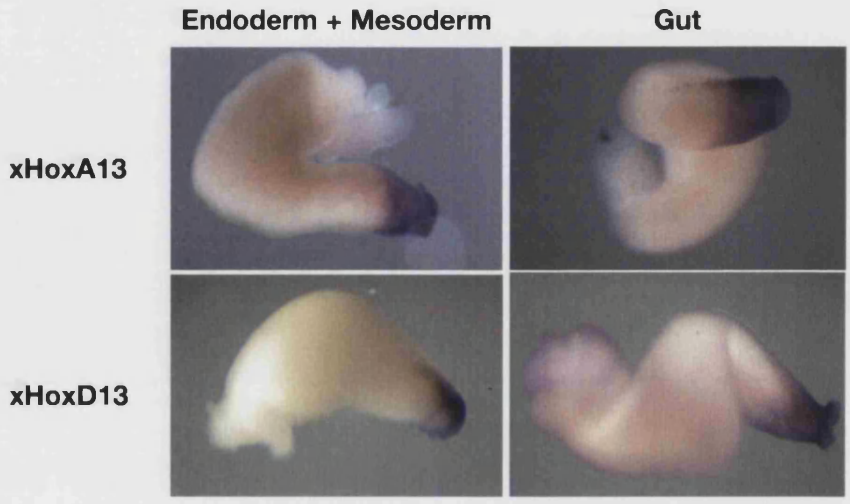


Figure 6.3 More posterior explants.
A) Diagram on the left shows how whole endoderm explant were normally made. This method was modified to include more posterior tissue to see if *xHoxA13* & *xHoxD13* might be expressed in more posterior tissue. The new cutting positions are shown on the right diagram. B) Shows the *in situ* hybridisation for *xHoxA13* & *xHoxD13* with the new more posterior explant and stage 42 gut. The more posterior explant shows clear expression of the *Hox* genes *xHoxA13* and *xHoxD13*.

The sections from the whole mount *in situ* gut provided us with several new pieces of information regarding the expression pattern of the *Hox* genes (Figure 6.4). *xHoxA9* and *xHoxD9* due to weak staining, did not give a clear indication as to where they were expressed, endoderm or mesoderm. *XhoxD10* and *xHoxC12* were clearly expressed in the mesoderm as the staining was very strong in the mesoderm (a thin layer around the outside of the gut) with no staining at all in the endoderm (inside cells of the gut).

Analysis of the expression pattern of *xHoxA13* and *D13* however was not as straightforward. At a glance it would seem that the expression was only in the mesoderm. However a closer look at the sections show that the inside endodermal cells may also show weak staining. It is interesting to note that in these sections we saw the strength of the staining being strongest at the outside of the gut where it is most exposed, gradually getting weaker towards the centre of the endoderm.

We believe that such outside to inside graduated staining patterns can be indicative of penetration problems with regard to the probe or the antibody. The strength of an *in situ* staining is directly correlated to the amount of probe hybridised at that particular area. If the probe is having difficulty penetrating the tissue then it follows that the highest amount of probe available for hybridisation would be on the outside most exposed side of the tissue sample gradually getting less toward the center of the sample. Because of this unequal distribution of probe, we would get a graduated stain, even if the gene of interest was expressed to an equal extent across the depth of the sample.

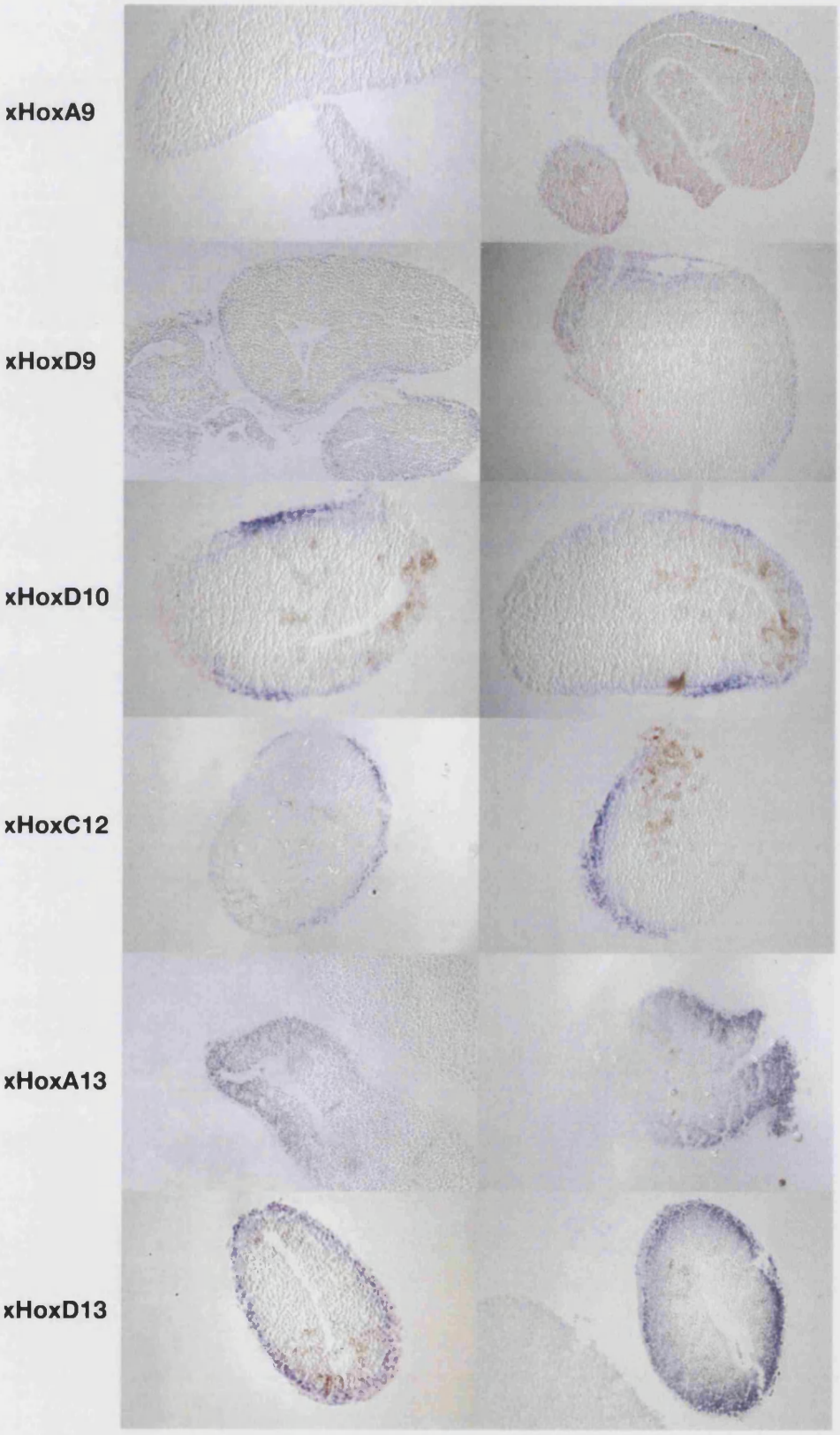

xHoxA9
xHoxD9
xHoxD10
xHoxC12
xHoxA13
xHoxD13

Figure 6.4 Wholemount *in situ* sections.
10 µm transverse wax sections cut following whole mount *in situ* hybridisation with stage 42 gut for *xHoxA9, xHoxD9, xHoxD10, xHoxC12, xhoxA13 and xHoxD13*. *xHoxA9* and *xHoxD9* did not show any staining probably due to the weak whole mount in situ observed earlier. *xHoxD10 and XhoxC12* showed a distinct mesodermal staining. *xHoxA13* and *xHoxD13* have the strongest expression in the mesoderm but also showed a graduated staining that gets weaker as towards the center of the sample.

Thus in order to get around this penetration problem, we need to increase the amount of tissue exposed. An obvious way to achieve this would be by sectioning the gut sample prior to *in situ*. We attempted this with wax, cryosection and vibratome but were not successful in obtaining a good *in situ* stain. Each of the methods had its own drawbacks and problems. We found the wax sectioned samples to have a greater tendency to develop non-specific staining, even when levamisole was included in the reaction mixture. Cryosectioned samples had a tendency to fall off the slide during the *in situ* process. We tried using various different slides: superfrost, superfrost gold and polylysine coated slides but none of them were able to keep the cryosectioned samples from falling off. The vibratome sections also had a tendency to be lost in the process of *in situ*. However this was because vibratome samples can not be mounted on a slide prior to *in situ* and must instead be collected in a 5ml tube and treated as a whole mount sample during *in situ*. Since the sections were small they tended to get sucked up by the glass pipettes during solution changes.

Due to all these difficulties performing *in situ* hybridisationon sectioned samples we decided to take an alternative route to increasing the amount of exposed surface area in the sample. The main problem with wholemount *in situ* of *xHoxA13* and *D13* was that we were not sure that the probe was capable of penetrating all the way to the endodermal cells inside the gut. Thus, to address this we decided to hemisect the gut prior to *in situ* in order to expose the inside cells of the gut. This would allow penetration of the tissue from both the outside and the middle of the gut, aiding the probe in reaching the endodermal cells located inside the gut. The cut was made longitudinally, dividing the gut into two half cylinders.

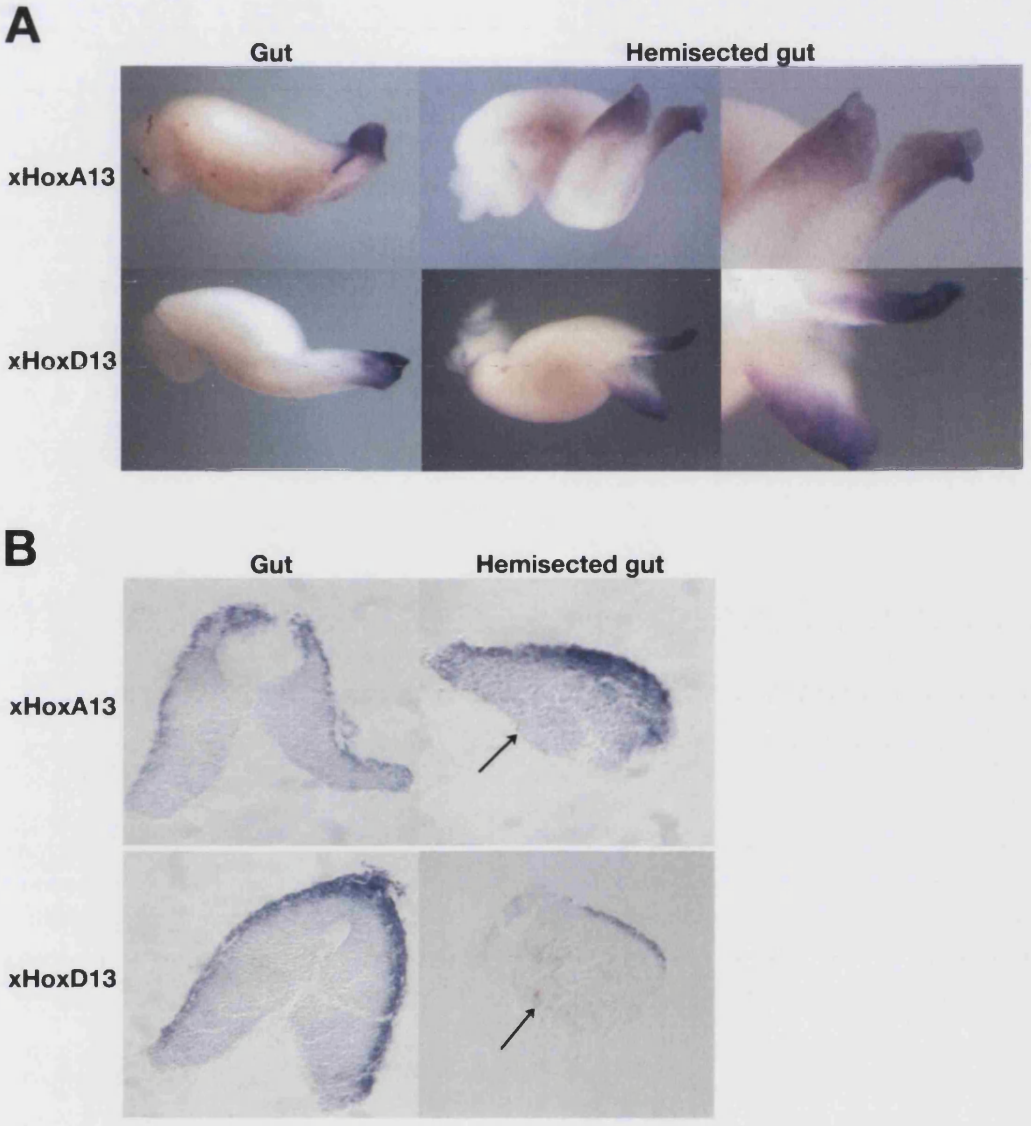


Figure 6.5 *In situ* on hemisected gut.
A). Results for *in situ* hybridisation where the posterior of the gut was hemisected after fixation but prior to *in situ*. B) The gut and hemisected gut was wax embedded and cut transversally at 10 µm. Black arrows on the hemisected gut figures indicates the inside exposed edge.

We tried hemisecting the gut before and after fixation and found that gut hemisected prior to fixation in MEMFA tend to curl. This was unsuitable for our purposes as it would make it difficult to perform traverse sections on the curled tissue, making it impossible to conclusively determine if the signal was in the endoderm or mesoderm. The gut remained relatively straight if cut after fixation in MEMFA. However care must be taken when

hemisecting the gut after fixation as the tissue is more brittle and more prone to breaking. Our previous *in situ* hybridisation with the whole gut had shown that *xHoxA13* and *xHoxD13* were only expressed in the posterior of the gut. Thus there is little benefit in hemisecting the gut all the way from the anterior to the posterior compared to just hemisecting the posterior. Hemisecting the posterior is much easier as we only need to split a much smaller part of the gut, minimising the chance of the gut breaking and becoming unusable.

Once hemisected the gut was then collected in a 5 ml tube and processed for *in situ* as a whole mount sample. Result of the *in situ* with *xHoxA13* and *D13* with whole gut and hemisected gut are shown in figure 6.5A. Looking at the hemisected gut it seems that these two genes might be expressed in both layers. However as was previously discussed, this method does not have enough resolution to conclusively say one way or the other. Thus we decided to process the hemisected gut, embed them in wax and perform transverse 10μm sections to see where the signal was.

Results from sectioning the hemisected gut was unexpected. As we can see from the photographs in figure 6.5B, hemisecting the gut and increasing the amount of exposed surface did not seem to change the expression pattern previously observed with *xHoxA13*. the strong dark staining was still only on the mesoderm layer around the outside of the gut and gradually getting weaker towards the endoderm. We see no increased staining in the endoderm where the cut was made. This means that we now know conclusively that the staining was real and not a result of the probe accumulating on the exposed side as there was no staining in the endodermal cells.

With *xHoxD13,* expression in the hemisected gut was only seen in the outside cells of the mesoderm. No expression was seen in the endodermal cells. This then indicates that the earlier staining in the endodermal cells might be non-specific staining and we can conclusively say that *xHoxD13* is expressed in the mesoderm only.

VI.4. Discussion

We found that the *Hox* genes *xHoxD9, xHoxD10, xHoxC12, xHoxA13 and xHoxD13* that were shown previously to be expressed regionally in the whole embryo to be expressed in the endo+meso explants but not on the endoderm-only explant. We must note however that a more posteriorly cut endo+meso explant was needed to see expression of *xHoxA13* and *xHoxD13*. This raises a few questions as to whether or not they are involved in endoderm specification. This posterior expression indicates that they might be expressed at the proctodeal level, where other genes are expressed in the endoderm. This region of the endoderm is a different territory from the future intestine (Chalmers and Slack, 2000).

However, it is possible that *xHoxA13* and *D13* might still be involved in endoderm development. It is conceivable that *xHoxA13* and *D13* can induce the formation of a posterior gradient in the embryo which in turn might pattern the endoderm. We will discuss further the possible role for *XhoxA13* and *D13* in endoderm development in the next chapter.

We were unable to obtain a clear expression pattern for the *Hox* gene *xHoxA9* in either the explant or gut samples. The staining we found on isolated gut and explants for this gene might be attributed to non-specific hybridisation, which was consistent with observations made on the sectioned gut samples. This was unexpected as previously *xHoxA9* has been shown to be expressed in the posterior isolated gut of stage 42 and later embryos (Lombardo and Slack, 2001). We are confident that the non-specific staining was not due to the probe as we tested with probes made from 3 separate transcription reaction and found all of them gave the expected pattern in control stage 35 embryos whilst showing non-specific background staining in the gut and endo+meso explant samples. There is the possibility that we might be able to obtain a staining in the gut and endo+meso explants if we developed the in situs for longer. *Hox* genes are expressed in very low levels and as such *in situ* for *Hox* genes usually requires a relatively longer development time. At the moment we have tried

development of up to 48 hours. We had considered and attempted longer development times but the non-specific staining became much too strong for this to be useful.

There is also the possibility that the expression levels of *xHoxA9* might have simply dropped off by stage 42, accounting for the faint, almost background like expression seen in our experiments. To test for this we would need to do RT-PCR with *xHoxA9* on different stages of embryos. RT-PCR would be more appropriate than in situ for this purpose as it is a more quantitative method. This method would also help to find the highest level of *xHoxA9* expression, indicating the likely stage at which it is involved in the development of the embryo.

We also attempted to confirm the location of the expression of the *Hox* genes by sectioning the gut post *in situ*. We can conclusively say that three of the *Hox* genes, *xHoxD10, xHoxC12* and *xHoxD13*, are expressed exclusively in the mesoderm. We were not able to confirm the location of *xHoxD9* expression, as the weak wholemount staining was not visible in the 10 µm wax sections. *xHoxA13* was definitely expressed in the mesoderm and it is possible that there is also some graduated staining in the endoderm. Initially we thought that the graduated staining pattern found with these probes might be due to limited penetration. However looking at *in situ* results with hemisected guts, where the inside endodermal cells were similarly exposed as the mesoderm cells on the outside we can be sure that this was not the case and that the observed pattern was genuine

Graduated staining is usually indicative of a gradient, normally observed with morphogen gradients involving proteins. Our current result however was showing graduated levels of *xHoxA13* RNA. A gradient involving RNA instead of protein has been known to exist. In chick and mouse *FGF8* mRNA is seen to form a posterior gradient from the tail bud and is involved in the formation of somites (Delfini et al., 2005; Dubrulle and Pourquie, 2004). This *FGF8* gradient is formed as a result of axis elongation and RNA decay. *FGF8* mRNA expression is restricted to the tail bud only.

During axis elongation, the tail bud cells divide resulting in a posterior extension of the embryo. The descendants of the tail bud inherits the FGF8 mRNA and due to polarised axis elongation take up an increasingly more anterior position in the embryo. Thus taking into account mRNA decay it can be seen how more anterior cells (older descendants) would have a lower level of *FGF8* mRNA compared to more posterior cells (newer descendants), creating a posterior gradient of *FGF8* mRNA (Dubrulle and Pourquie, 2004). However a similar mechanism is unlikely to be the cause of the *xHoxA13* gradient as it involves two germ layers. The graduated expression in *xHoxA13* seems to be strongest from the mesoderm getting weaker towards the inner endodermal cells. Thus since the endodermal cells are unlikely to be derived from the mesodermal cells, the gradient could not be formed using the same mechanism as the *FGF8* gradient.

There is no clear explanation as to why the level of expression was so much lower in the endoderm compared to the mesoderm. It could be the endoderm has a lower threshold of *xHoxA13* response that it only needs to express it at low amounts. It is also possible that *xHoxA13* is acting differently in these two layers, hence the different level of expression. To address this a study into the activity of downstream targets of *xHoxA13* would be needed through either differential RNA analysis or microarrays.

In conclusion, the discovery that the regionally expressed *Hox* genes *xHoxD9, xHoxD10, xHoxC12, xHoxA13* and *xHoxD13* are expressed in the endo+meso explant is lends support to their proposed role in endoderm specification. The observation of the strong expression of *xHoxD10, xHoxC12, xHoxA13* and *xHoxD13* in the mesoderm means that these genes are expressed in the right germ layer to provide positional information to the mesoderm along the A-P axis, which in turn could allow the mesoderm to pattern the endoderm accordingly.

VII. *Hox* overexpression

VII.1. Introduction

Hox genes, a subset of the homeobox genes, have been shown to be involved in the A-P patterning of the mesoderm and ectoderm (Krumlauf, 1994; McGinnis and Krumlauf, 1992; Pearson et al., 2005). There is some evidence suggesting that they might be involved in the A-P patterning of the endoderm as well. The *Hox* genes have been found to be expressed in a nested, overlapping patterns in the mesoderm of developing gut in chick (Roberts et al., 1995; Sakiyama et al., 2000; Sakiyama et al., 2001; Yokouchi et al., 1995). The nested expression pattern is particularly relevant to endoderm development as the boundaries of the expression pattern of some of these mesodermaly expressed *Hox* genes have been shown to align with the morphological borders of different gut regions (Grapin-Botton, 2005). This is especially true for the 5' members of the *Hoxa* and *Hoxd* clusters (paralogues 9-13) in chick (Roberts et al., 1995).

Previous heterologous recombination experiments in chick, mouse and *Xenopus* have shown that the mesoderm is important in patterning the endoderm along the A-P axis (Horb and Slack, 2001; Kumar et al., 2003; Wells and Melton, 2000). To pattern the endoderm along the A-P axis the mesoderm needs to be told of its position along the A-P axis. Thus this nested expression of *Hox* genes in the mesoderm puts them in the ideal position for providing positional information to the mesoderm along the A-P axis.

Indeed the misexpression of *Hoxd-13* in chick has been shown to be able to induce a hindgut fate on midgut tissue (Roberts et al., 1998). This is further supported with murine transgenic experiments where inactivation or misexpression of the *Hox* genes result in abnormal gut development (Pollock et al., 1992; Wolgemuth et al., 1989). In particular, loss of expression of *Hoxa-13* and *Hoxd-13* have resulted in the alteration of

muscle layers of the sphincter, which is consistent with a partial anterior transformation of this region (Kondo et al., 1996; Warot et al., 1997).

In *Xenopus* members of the *Abdominal B*-type *Hox* genes have been shown to be regionally expressed in the mesoderm (Lombardo and Slack, 2001). Even though in *Xenopus* the boundaries have not yet been aligned with morphological boundaries, it still raises the possibility that, similar to that observed in chick and mouse, *Abdominal B-type Hox* genes might be involved in the development of the endoderm. We aimed to overexpress some of these *Hox* genes and study their possible roles and function in endoderm development.

An interesting feature of the *Hox* genes is that more posterior *Hox* tend to be dominant over the anterior *Hox*. Typically, if a posterior *Hox* gene is expressed ectopically at a more anterior position it results in that segment of the animal taking on a more posterior fate. However if an anterior *Hox* gene is expressed at a more posterior position, the tissue is not respecified towards a more anterior fate. This feature is known as "posterior dominance" (Manak and Scott, 1994).

Due to this we decided that it would be most informative to start by overexpressing the two most posterior *Xenopus Hox* genes, *xHoxA13* and *xHoxD13*. These genes were likely to be dominant and as such more likely to respecify anterior tissue yielding a visible abnormal phenotype. We expected that an overexpression of *xHoxA13* or *D13* in the whole embryo would lead to the posteriorisation of the gut, indicated by the expansion of *Xcad2* expression and down regulation of *Xlhbox8*.

VII.2. Materials and Methods

VII.2.1. Generating mRNA for injection

The mRNAs of *xHoxA13* and *xHoxD13* were generated by linearising the plasmids using NotI followed by a transcription reaction using SP6 mMessage machine kit (Amersham). The *GFP* mRNA was generated from ApaI linearised plasmid which was then put in a transcription reaction using

T7 mMessage machine (Amersham). Once obtained, the mRNA of interest was injected at the appropriate concentration in 4.6nl of solution using a Drummond injector. A total of 500pg of *GFP* mRNA was also injected along with the *Hox* mRNA. This coinjection allows us to trace the location of cells that have taken up the mRNA. For 4-cell dorsal injection the *GFP* marker should be clearly visible along the notochord and in the head structure.

VII.2.2. Cryosection

To observe the *GFP* signal in sections the embryos need to be fixed in 4%PFA for 2-3 hours at 4°C. Once fixed they are then washed twice in PBSA before being processed for cryosection. Cryosection is the preferred sectioning method here as it best preserves the fluorescence signal. The preparation for cryosection involve incubation in 15% sucrose and 30% sucrose both overnight at 4°C before embedding in OCT (Sigma).

The embedded samples were cryosectioned at -20°C with 15 µm per section. The sections were put on polylysine slides (Fisher) and allowed to dry at room temperature for approximately 3-4 hours. The slides were then washed with PBSA again twice for 5 minutes each to wash off the excess OCT before finally being mounted using Gelmount (Biomeda). Once mounted in Gelmount the *GFP* signal should be stable for a few months at room temperature.

VII.3. Results

VII.3.1. *xHoxA13* and *xHoxD13* overexpression

Overexpression in *Xenopus* can be done through two methods: RNA injections or transgenics. Each of these methods has their own advantages and disadvantages. RNA injection is clearly quicker to perform and all of the injected embryos would express the mRNA. However there is no temporal regulation of expression: translation commences shortly after injection and continues thereafter. The mRNA will eventually decay and its activity will terminate. Transgenics may enable permanent or regulated trasngene

expression but only a fraction of the injected eggs would express the integrate the injected plasmid and express the genes.

One of the advantages of working with *Xenopus* is that we have access to a complete fate map of the embryo (Dale and Slack, 1987; Lane and Sheets, 2002). Using this information we can inject specific blastomeres in the early embryos to target a particular set of tissues. This also gives us flexibility, for example if we need to target a different set of tissues we would only have to inject a different blastomere. In transgenics to target a different tissue we would have had to make a new construct driven by a different promoter. Based on these considerations we decided that it would be more effective to use RNA injections in establishing a possible role for *xHoxA13* and *D13* in the specification of the endoderm.

Both the recently completed (Lane and Sheets, 2002) and the original (Dale and Slack, 1987) fate maps of *Xenopus* indicate the same blastomeres that contributes to the endoderm and mesoderm. However for the sake of clarity we would like to note that in this thesis we are still using the traditional dorsal/ventral nomenclature (Dale and Slack, 1987) and not the recently proposed rostral/caudal nomenclature (Lane and Sheets, 2002).

VII.3.2. Dorsal Injection

Before testing if the *Hox* has any effect on endoderm specification, we decided to confirm that the mRNAs for *xHoxA13* and *xHoxD13* were functional by injecting them to the dorsal side of a 4-cell stage embryo. Overexpression of posterior *Hox* genes on the dorsal side of an embryo is known to suppress development of the anterior/head structure (Pownall et al., 1996). Location of the injection are shown in Figure 7.1 below.

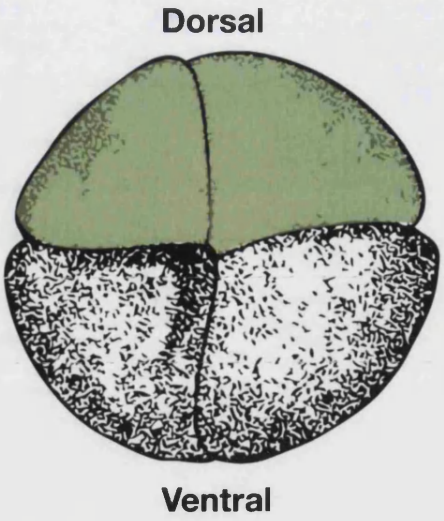
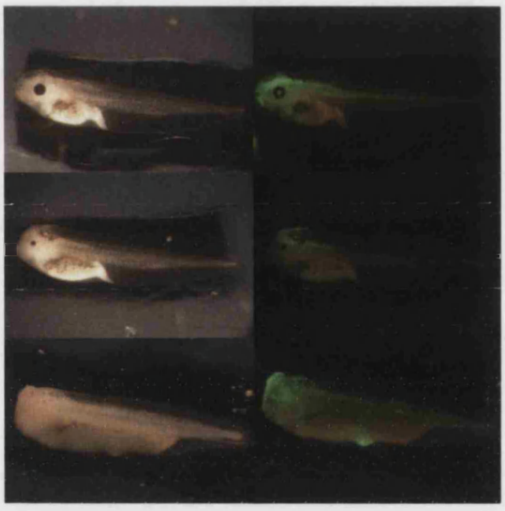


Figure 7.1 Top view of 4-cell stage embryo.
Figure above shows the difference between dorsal and ventral blastomeres at 4-cell stage. The injection for positive control is done on the dorsal blastomeres, highlighted in green above.

Dorsal injections with 2ng total *xHoxD13* resulted in one of two different phenotypes: a complete loss or reduction in the size of the eyes (Figure 7.2A), both of these representing a reduction in anterior/head structures. Approximately 76.2% of the embryos injected with 2ng of *xHoxD13* showed either one of these phenotype, confirming the biological activity of the *xHoxD13* mRNA.

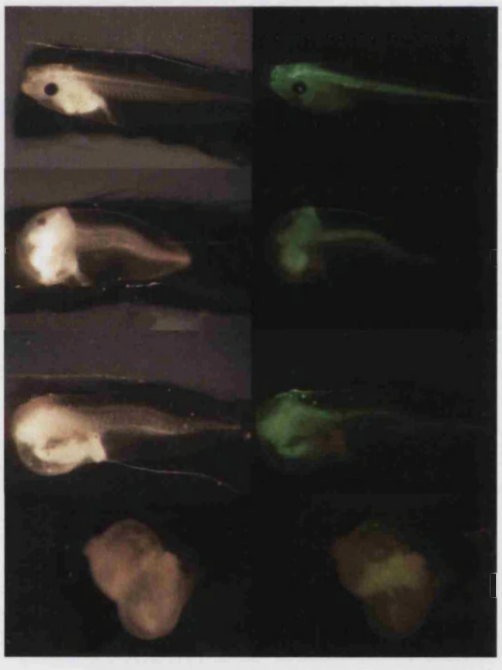
Our initial injections of 1ng total *xHoxA13* mRNA resulted in most (>90%) of the embryos showing a severely deformed phenotype extremely shortened with no visible anterior structures (Figure 7.2B). Initially we suspected that this might be due to toxic chemicals (e.g. phenols) present in the RNA preparation. However considering that we still observed a low number of embryos with the less severe head/eye suppression we concluded that these deformed embryos might just be a very severe form of anterior/head suppression.

A



Phenotype	GFP (2ng total)	xHoxD13 (2ng total)
Normal	44 (100%)	10 (23.8%)
Reduced Eyes	0 (0%)	15 (35.7%)	
Eye Loss	0 (0%)	17 (40.5%)

B



Phenotype	GFP (2ng total)	xHoxA13 (1ng total)	xHoxA13 (500pg total)
Normal	63 (100%)	0 (0%)	10 (16.1%)
Eye Suppression	0 (0%)	1 (1.8%)	18 (29.1%)
Head Suppression	0 (0%)	4 (8.0%)	14 (24.5%)
Extreme Head Suppression	0 (0%)	47 (90.3%)	15 (30.3%)

Figure 7.2 Dorsal injections of *xHoxA13* & *D13*.
A) *xHoxD13* injections in the dorsal blastomeres leads to suppression of the eye. Three different phenotypes were observed: normal, reduced eyes and complete eye loss. The numbers obtained with each phenotype is shown on the table to the right with the percentages in brackets. B) *xHoxA13* injections in the dorsal blastomeres leads to suppression of the eye and head. There were four phenotypes observed: normal, eye suppression, head suppression, extreme head suppression. The numbers obtained with each phenotype is shown on the table to the right with the percentages in brackets.

To confirm this we did injections with a lower amount of mRNA (500pg). If indeed this was a more severe phenotype of *xHoxA13* overexpression then we expected to get more of the less severe phenotype (e.g eye or head suppression) with lower amounts of *xHoxA13*.

Indeed when 500pg of *xHoXA13* was injected only 30.3% of the embryos were severely deformed. A majority of the injected embryos 53.6% showed either eye or head suppression. This then confirmed that the phenotypes observed were due to the biological activity of *xHoxA13* and not due to toxicity.

VII.3.3. *xHoxD13* ventral injections

After confirming that both the *xHoxA13* and *D13* RNAs had biological activity, we decided to target mesoderm and endoderm. Both layers were targeted as there was an indication that *xHoxA13* might normally be expressed in both endoderm and mesoderm and not mesoderm only. Also by targeting both these layers we could do the injection at an earlier stage, making it easier and faster. Injections were made on the ventral vegetal side of an 8-cell stage embryos (Figure 7.3). We injected both the left and the right blastomere to ensure that most of the gut is affected.

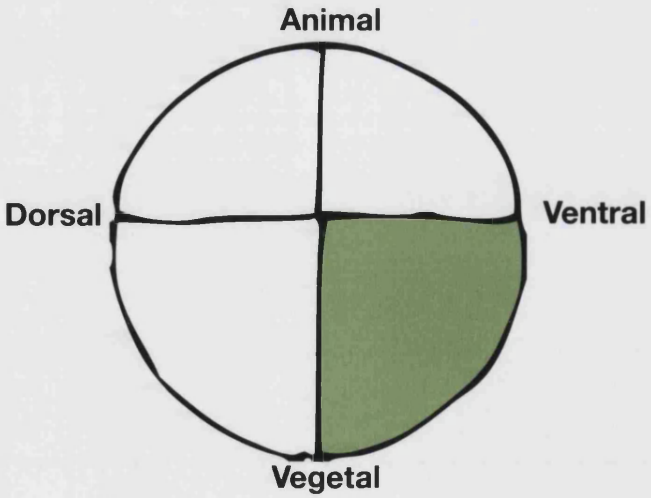


Figure 7.3 Diagram for 8-cell stage injection.
Side view of an 8-cell stage embryo. Location for the injection of the *xHoxD13* and *A13* is highlighted in green.

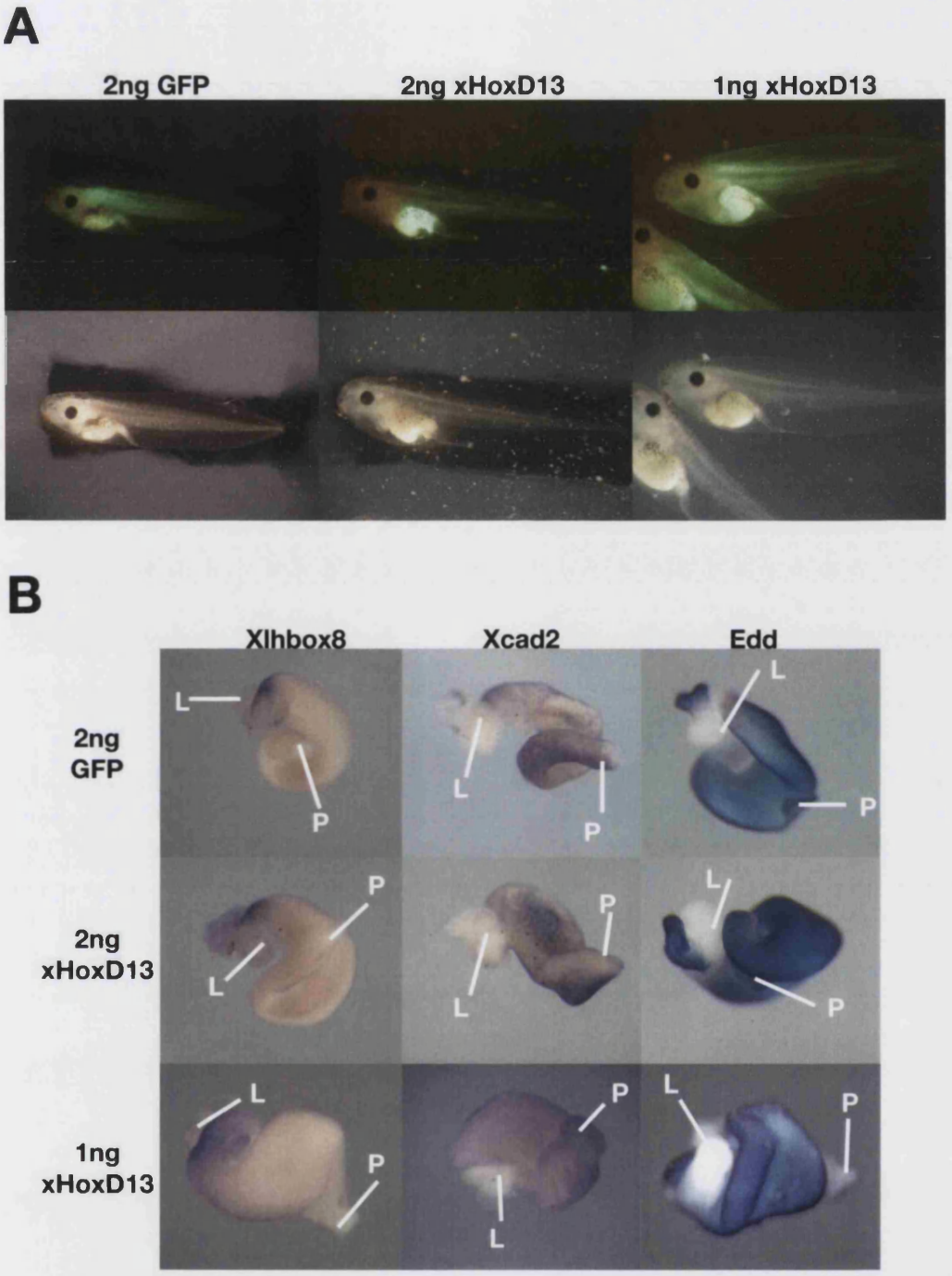


Figure 7.4 *xHoxD13* 8 cell ventral injections.
A) Shows the whole embryo for control injections with 2ng *GFP* as well as the experimental injections with 2 & 1 ng *xHoxD13*. The *xHoxD13* injected embryo looked similar to control *GFP* injected embryos. B) *In situ* for *Xlhbox8*, *Xcad2* and *Edd* with guts from the injected embryos. Highlighted in the panels are positions of liver bud (L) and proctodeum (P). Here we can see that the patterning of the gut is not affected by *xHoxD13* overexpression as the pattern with *Xlhbox8* and *Xcad2* remains similar to control *GFP* injected embryos.

We decided to do the injections at various concentrations to see which would have an effect on the development of the endoderm. Again as before *xHoxA13* mRNA were coinjected with 500pg of *GFP* mRNA to allow visualisation of affected embryos, thus confirming that the correct blastomere had been affected. Results for the injection of *xHoxD13* at 1 and 2 ng as well as the 2ng *GFP* control injection can be seen in Figure 7.4.

There was no visible difference between the *xHoxD13* injected embryos compared to the *GFP* injected embryos. *xHoxD13* did not seem to affect the normal development of the gut. This observation was confirmed when we did *in situ*s for *Xlhbox8*, *Xcad2* and *Edd* with guts isolated from the injected embryos. In the *xHoxD13* injected embryos *Xlhbox8* was still expressed in the anterior of the gut whilst *Xcad2* was expressed throughout the posterior of the gut with a distinct lack of staining in the anterior. This expression pattern was similar to that of *GFP* injected embryos which confirmed the normal expression pattern of both these markers. Thus, based on this it appears that ventral vegetal injections of *xHoxD13* at 8-cell stage does not affect endoderm specification.

VII.3.4. *xHoxA13* ventral injections

We also injected a series of concentrations of the *xHoxA13* mRNA towards the ventral vegetal side of 8-cell stage embryos. Again 500pg of *GFP* mRNA was coinjected with the *xHoxA13* mRNA to help visualise the location of the injections and thus confirm that the correct cells had been affected. Results for the injections of *xHoxA13* at 1ng and 500pg as well as the control 2ng *GFP* injection can be seen in Figure 7.5.

A clear phenotype with the *xHoxA13* injections were seen when at least 500pg of mRNA was injected (Figure 7.5A). In approximately 30-40% of the embryos we observe a bubble forming around the gut of these embryos. The gut itself seems thinner and lacks coiling. These observations indicated that the normal morphological movements that takes place in the development of the gut were affected by the overexpression of *xHoxA13*.

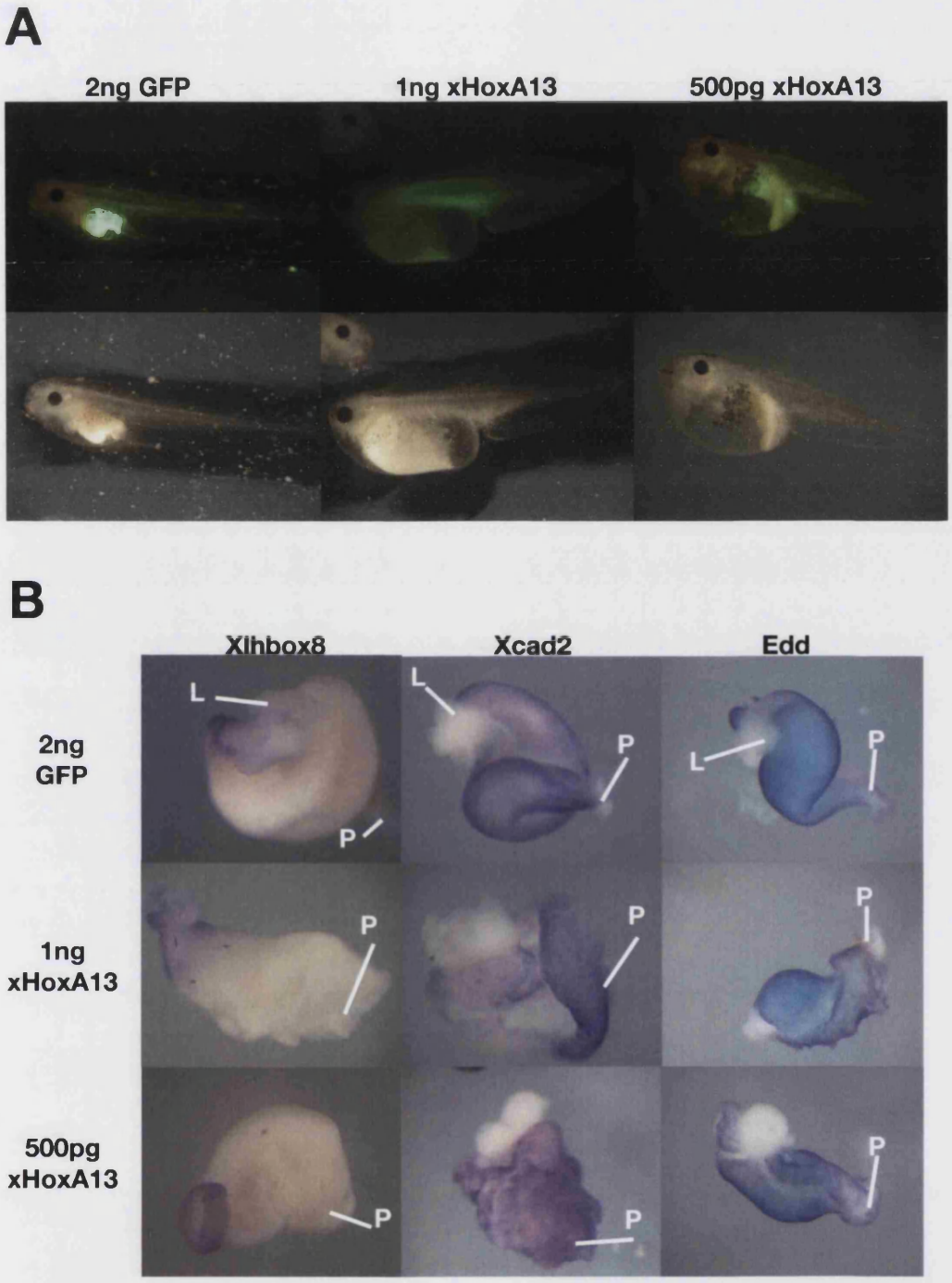


Figure 7.5 *xHoxA13* 8 cell ventral injections.
A) Shows the whole embryo for control injections with 2ng *GFP* as well as the experimental injections with 1ng & 500 pg *xHoxA13*. The *xHoxA13* injected embryo shows an expanded gut that seems to have failed to coil properly. B) *In situ* for *Xlhbox8*, *Xcad2* and *Edd* with guts from the injected embryos. Highlighted in the panels are positions of liver bud (L) and proctodeum (P). Here we can see that the patterning of the gut is not affected by *xHoxA13* as the pattern with *Xlhbox8* and *Xcad2* remains similar to control *GFP* injected embryos despite a clear difference in morphology.

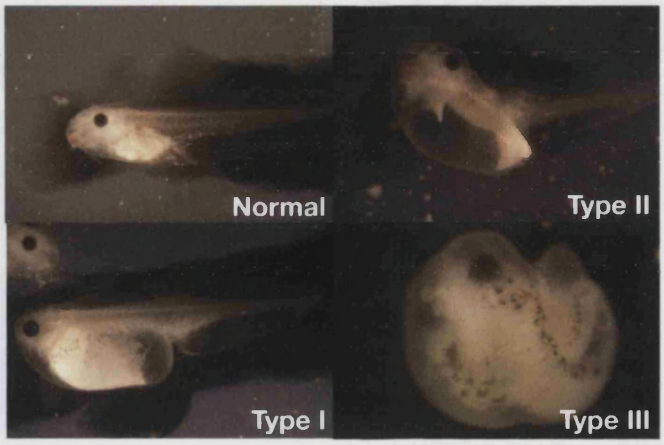
To see if the *xHoxA13* overexpression affected the specification of the endoderm as well as the morphology we followed up by performing *in situ*s for *Xlhbox8*, *Xcad2* and *Edd* on the isolated guts of the injected embryos. Unexpectedly results from these *in situs* showed that the normal specification of the endoderm was maintained in these deformed guts. We can see that *Xlhbox8* is still expressed in a narrow band in the anterior of the gut and that *Xcad2* is still expressed in the posterior of the gut with a distinct lack of expression in the anterior of the gut (Figure 7.5B).

Thus the results from the ventral vegetal injection at 8-cell stage indicates that *xHoxA13* does not have any effect on the regional specification of the endoderm. Its visible effects in inhibiting coiling of the gut may indicate that it is involved with morphological movements that takes place later than endoderm specification in the development of the *Xenopus* endoderm (Chalmers and Slack, 1998). Also we had only observed the gut deformation when an oedema was present. This then raises the possibility that the influx of water into the embryo might also have gone into the gut, which in turn would cause morphological defects.

In both *xHoxA13* and *D13* ventral vegetal injection at 8-cell stage did not yield any respecification of the endoderm. However, according to the fate map the dorsal vegetal blastomere also contributes to some of the endoderm. We did not inject this in our experiment because injection to the dorsal side might bring up the head/anterior suppression phenotype which in turn would complicate the observation. But in doing this we might not have filled the anterior dorsal and ventral regions that form the *Xlhbox8* domain with the *Hox* RNA. Since a posterior respecification, would manifest in downregulation of *Xlhbox8*, this incomplete delivery of RNA might result in a false negative result of no respecification.

VII.3.5. *xHoxA13* phenotype characterisation

To better understand the phenotype observed earlier with the ventral vegetal injections of *xHoxA13* to 8-cell embryos we needed to characterise it further. When we looked closer at the *xHoxA13* embryos, we observed 4 distinct phenotypes: normal and type I, II and III (Figure 7.6).



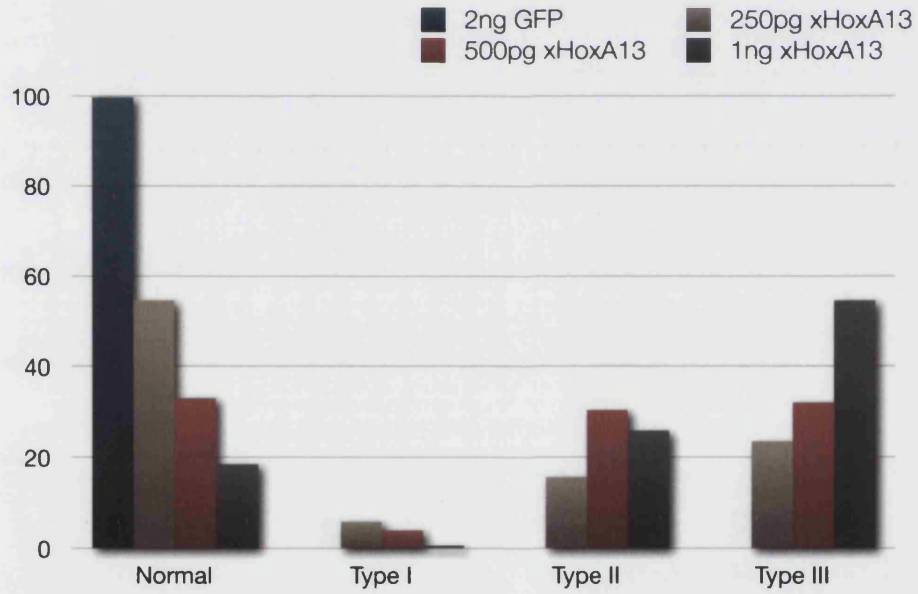


Figure 7.6 Classification of *xHoxA13* phenotypes.
We observed four different types of phenotypes observed when *xHoxA13* was injected at ventral vegetal blastomeres of 8-cell stage embryo. The photograph above shows examples of all four of the different phenotypes observed: normal, type I, II & III. Relative frequencies of each of the phenotypes observed for 2ng *GFP* and 1ng, 500 & 250 pg *xHoxA13* injections are shown in the graph below.

The normal phenotype is self explanatory, a control phenotype indicating that the embryo has developed normally. In the type I phenotype a bubble has formed around the gut. The bubble tend to be cylindrical, with the gut apparently attached to the bubble and thinner towards the posterior end. The bubble around the gut was less distended than type II phenotype, possibly indicating an incomplete malformation. Therefore we believe that this phenotype is the least severe abnormal *xHoxA13* phenotype.

Type II phenotype embryos had also developed a bubble surrounding the gut, however the bubble in these embryos was larger and was not cylindrical. The gut was not attached to the bubble as it was with the type I phenotype, but was instead flattened and attached to the dorsal side of the embryo. This was more severe than the type I phenotype as the oedema was larger and the gut was visibly more deformed.

The type III phenotype was the most severe of all the phenotypes. The embryos have been severely shortened with most of the structure not developing normally. This phenotype was more typical following injection of 1ng or higher amounts of *xHoxA13*. At this high concentration there is a possibility that the *xHoxA13* had reached toxic levels. Thus, this phenotype might be a result of having a significant number of cells dying due to toxicity caused by an excess amount of *xHoxA13* protein.

We did a series of injections with 250pg, 500pg and 1ng of xHoxA13 as well as 2ng *GFP* control. Again the xHoxA13 was coinjected along with 500pg *GFP* as a label. The relative frequencies of the different phenotypes can be seen in the graph at Figure 7.6. The frequencies of the phenotypes seem to be dependent on the amount of xHoxA13 injected.

From all the three phenotypes present, we decided to concentrate on the type II phenotype as this could be considered the most representative of the *xHoxA13* phenotype. Thus we decided to concentrate on the type II phenotype, which will be referred to simply as *xHoxA13* phenotype from this point. Looking at the relative frequencies of the phenotypes we decided that

the optimal amount of *xHoxA13* that needs to be injected was 500pg per embryo as it generates the highest number of the type II phenotype.

VII.3.6. Timing of phenotype

Previously, from looking at the gut in the whole embryo as well as *in situ* results we concluded that *xHoxA13* is most likely affecting gut morphogenesis and not specification. Since gut morphogenesis is a late event, that starts sometime between stage 40-41 (Chalmers and Slack, 1998), we thought it might be interesting to identify the time at which the *xHoxA13* injected embryos starts developing abnormally. As before, embryos were injected at the ventral vegetal side (Figure 7.3) of 8-cell stage embryo. The injected embryos were then cultured in Nam/10 and were checked daily. A sample of injected embryos (about 10) was taken each day and fixed in 4%FA. These samples would later be sectioned to help characterise the phenotype.

Observing the *xHoxA13* injected embryos we noted that the embryos develop normally up to stage 39. The bubble only developed on the *xHoxA13* injected embryos starting from around stage 41 (Figure 7.7). From this we were able to conclude that the *xHoxA13* is only visibly affecting the development of the endoderm between stage 39 and 41. This was consistent with our earlier analysis that *xHoxA13* affects gut morphogenesis, which is a late event, and not endoderm specification. However because of a lack of temporal control with RNA overexpression, we cannot rule out a possibility that the visible abnormal phenotype we see between stage 39 and 41 might be a manifestation of an earlier *xHoxA13* effect. The injected *xHoxA13* mRNA might have elicited its effects anytime between when it is injected and stage 39.

Once we established the timing, we then sectioned the fixed samples to see exactly how the gut development had been disrupted. Transverse sections of stage 41 *GFP* injected and *xHoxA13* injected embryos were made at 10μm per section. Photographs of these sections is shown below

in figure 7.8. We observed the epidermal bubble clearly in the *xHoxA13* injected embryos. Also we can clearly see a lack of coiling in the gut of *xHoxA13* injected embryos confirming our observations on the wholemounts.

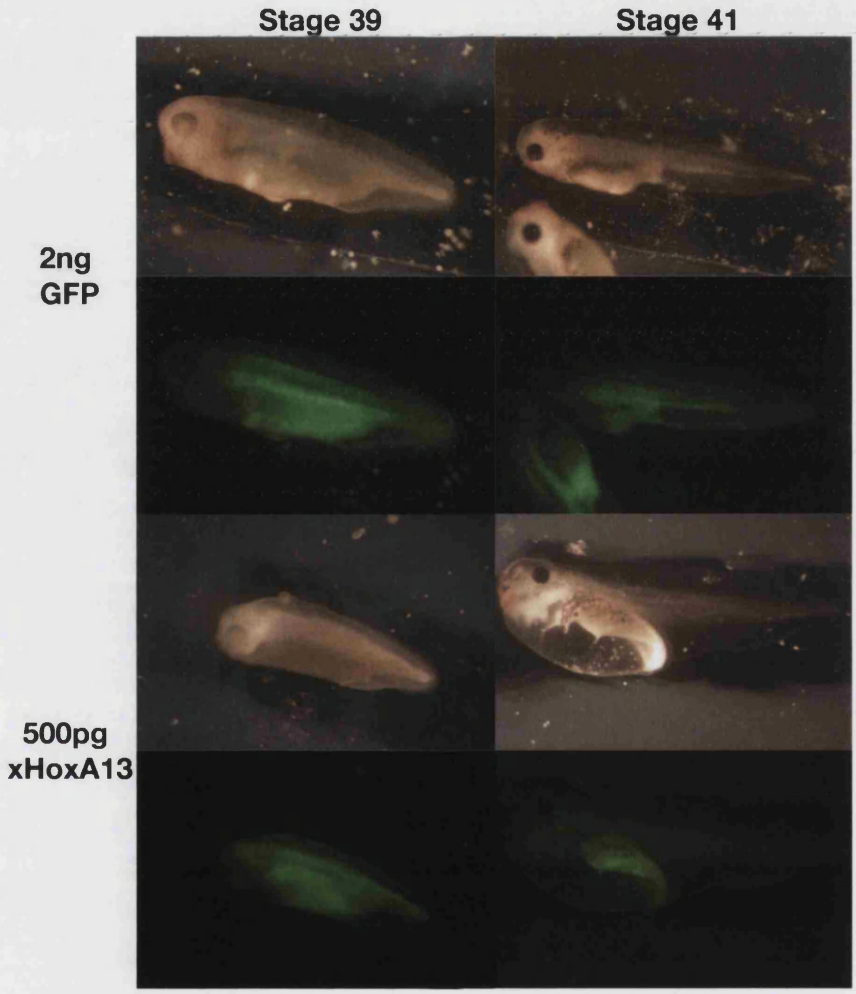


Figure 7.7 *GFP* and *xHoxA13* injected embryos at stage 39 & 41.
The *xHoxA13* injected embryos look normal at stage 39. At stage 41 however they have developed a bubble around the gut.

Observations of the sections also reveal that the inside of the endodermal mass contains a cavity. In the last three posterior sections of the *xHoxA13* embryos shown in Fig 7.8 we can clearly see the gap starting to develop and gradually getting larger. It would seem that the layer of endodermal cells in the *xHoxA13* embryos is quite thin, unlike in the *GFP*

injected controls where it forms a solid mass of cells. This is most evident with the posterior most section of the *xHoxA13* embryos.

There are several possibilities for the cause of this cavity in the middle of the endoderm. One is that somehow the cells in the middle of the gut have died off leaving the gap in the middle. However this is unlikely because we did not observe any necrotic cells in the sections. Thus considering that the gut cells seem to be thinning especially towards the posterior we believe it was more likely that the cavity was a result of abnormal morphogenetic movements instead of cell death.

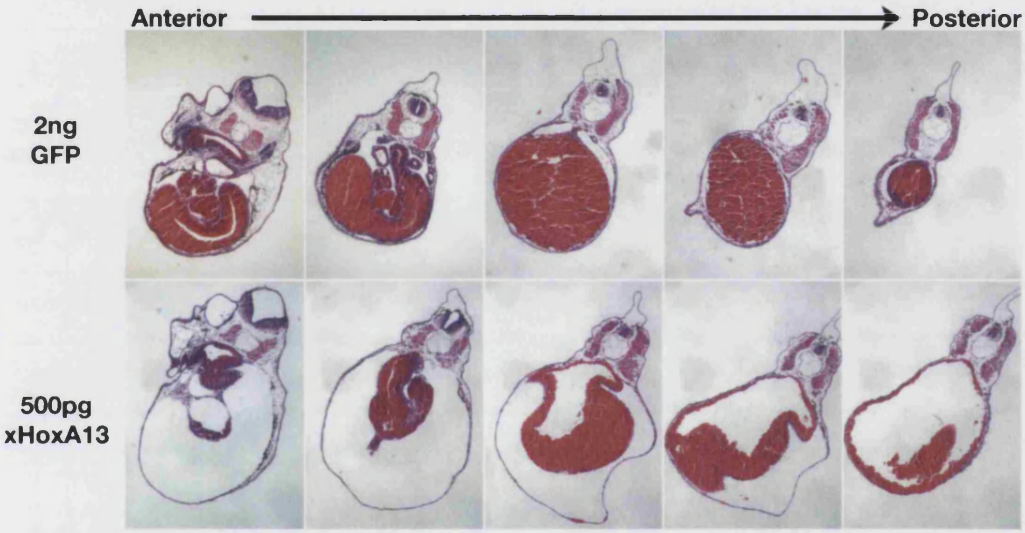


Figure 7.8 Transverse sections of *GFP* and *xHoxA13* injected embryos.
Figure above shows transverse sections of stage 41 embryos that have been injected with *GFP* and *xHoxA13*.

During normal development, two different cavities form and close in the embryo: the blastocoel, which is formed early and the archenteron, which is formed late. Considering that the *xHoxA13* phenotype occurs late, around stage 39-41, it is more likely that the cavity observed here is derived from the archenteron and not blastocoel. Previous study have shown that morphogenetic movements of the endodermal cells causes the archenteron to close around stage 38 before re-opening to form part of the gut cavity at stage 45 (Chalmers and Slack, 2000). Thus a disruption of morphogenetic

movements by *xHoxA13* might prevent the archenteron from closing, leading to the large cavity seen in the *xHoxA13* phenotype.

VII.3.7. Affected Cells, endoderm or mesoderm?

During our injections we injected on the ventral vegetal side of 8-cell stage embryo. According to the fate map, these blastomeres contribute to both the mesoderm and endoderm layer derivatives (Dale and Slack, 1987). The distribution of the injected cells is somewhat random and as such there is no way to control which layer would have taken the most of *xHoxA13* RNA.

This might explain the incomplete penetrance of the phenotype. The uneven uptake of the RNA by either layer could conceivably lead to different phenotypes being observed. For example the bubble that develops around the gut could be due to most of the *xHoxA13* mRNA being taken up by the endoderm or it could be by the mesoderm or it could be by both. The location of the effect is especially relevant for *xHoxA13* as it is normally expressed in both endoderm and mesoderm and as such may have an equal chance of affecting either layer when overexpressed. Thus to go further and understand the mechanism and causes of the phenotype it is important to see which germ layer is primarily affected.

In all of our injections we always coinjected with GFP mRNA. *GFP* here acts as a label, thus to see exactly where the *xHoxA13* has accumulated we would need to see the *GFP* on transverse sections of the affected embryos as whole mounts would not have enough resolution.

Photographs of the transverse cryossections of *GFP* and *xHoxA13* injected embryos can be seen in Figure 7.9. Looking at the *GFP* we can infer that the *xHoxA13* mRNA has been taken up by the endodermal cells of the gut as well as the somites which would indicate that both the mesoderm and endoderm have been affected.

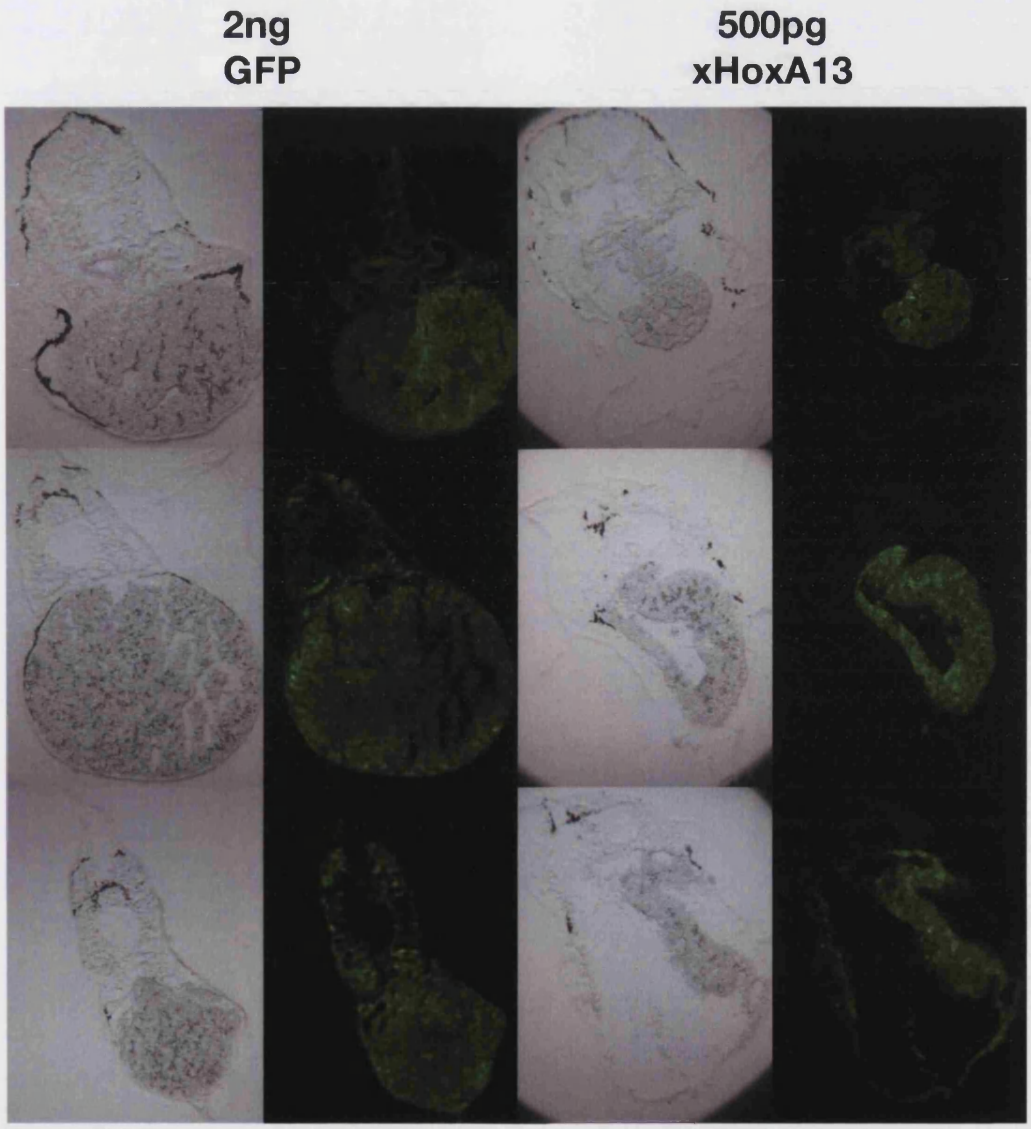


Figure 7.9 Transverse sections embryos injected with *GFP* and *xHoxA13*. *GFP* shows the location of cells that have taken up the injected RNA. As can be seen most of the *GFP* seen in the *xHoxA13* injected embryos are localised to the endoderm.

However with the *xHoxA13* injected embryos, the *GFP* signal seems to be found only in the endoderm. There was no signal coming from the somites. Thus it would seem that from this initial evidence that the *xHoxA13* was eliciting its effect in the endoderm and not mesoderm. We need to note however that It was difficult to determine whether or not *GFP* was expressed in the sphlanchnic mesoderm. This particular layer of cells is hard to detect in normal embryos and even harder to see in a deformed one.

VII.3.8. 32 cell injections

To test if it was the case that *xHoxA13* only needs to affect the endoderm to induce the phenotype, we went on to do injections on 32-cell stage embryos. This later stage injection should allow us to be more specific with our target cells compared to the 8-cell injections.

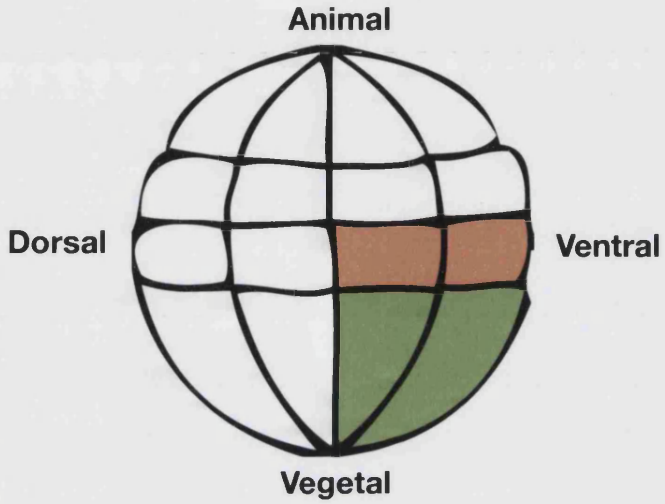


Figure 7.10 Diagram of 32-cell stage embryo.
Diagram showing the location of injections done on 32-cell stage embryos. The orange blastomeres indicates C3 and C4 blastomere and the green blastomere indicates D3 and D4 blastomeres.

According to the fate map the C3 and C4 blastomeres (orange blastomeres on Figure 7.10) mostly contribute to mostly mesodermal cells and the D3 and D4 blastomeres (green blastomeres on Figure 7.10) contribute to mostly endodermal cells (Dale and Slack, 1987). Thus by doing injections to either ventral C-tier (C3 and C4) or ventral D-tier (D3 and D4) blastomeres we can target the mesoderm or endoderm respectively. The total amount of mRNA injected was maintained at a total of 500pg per embryo for all of these injections. The volume of injections per blastomere was also kept at 4.6nl.

Injections to the C3 and C4 blastomeres with *xHoxA13* resulted in a normally developing embryo, and surprisingly, so did the injections to the D3 and D4 blastomeres (Figure 7.11). This then raised the possibility that both the mesoderm and endoderm is required to generate the phenotype.

To confirm this we decided to do injections to all four blastomeres on the ventral vegetal side of the 32-cell stage embryo (C3, C4, D3 and D4). The total amount of *xHoxA13* mRNA was again kept at 500pg per embryo and was injected at 4.6nl per blastomere. Results from this injection showed that when all four of the blastomere were injected we were able to generate the phenotype again. This then indicated that both the mesoderm and endoderm needs to take up the *xHoxA13* mRNA to be able to generate the phenotype.

This was very interesting considering that our earlier observation showed the *GFP* in the 8 cell stage *xHoxA13* injections were mostly localised to the endoderm. There are several possible explanations as to the lack of apparent *GFP* staining in the mesoderm of *xHoxA13* embryos. It is possible that the mesodermal cells that have taken up *xHoxA13* mRNA might have mixed in with the endoderm thus disrupting the gut development

Similarly possible is that the mesodermal cells might have been more sensitive towards *xHoxA13*. Thus the cells that have taken up *xHoxA13* might have been killed off leading to the disruption of interaction between endoderm and mesoderm. This would then result in the disruption of gut morphogenesis leading to the deformed gut in the *xHoxA13* phenotype. In this case the *GFP* signal would also be missing from the mesoderm.

To be able to test for these two possible mechanism we would need to be able to label the mesodermal and endodermal cells differently. This would require using two different labels to label each type of cells. Thus far in our experiments we have used *GFP* to label which cells have taken up the mRNA. There is another marker that will work in a similar fashion, rhodamine-dextran. Rhodamine-dextran is a fluorescent chemical molecule giving out a red fluorescence which is very different from the green emitted by *GFP* thus allowing for distinction. Like *GFP,* however, it can also label cells that have taken up mRNA in an injection.

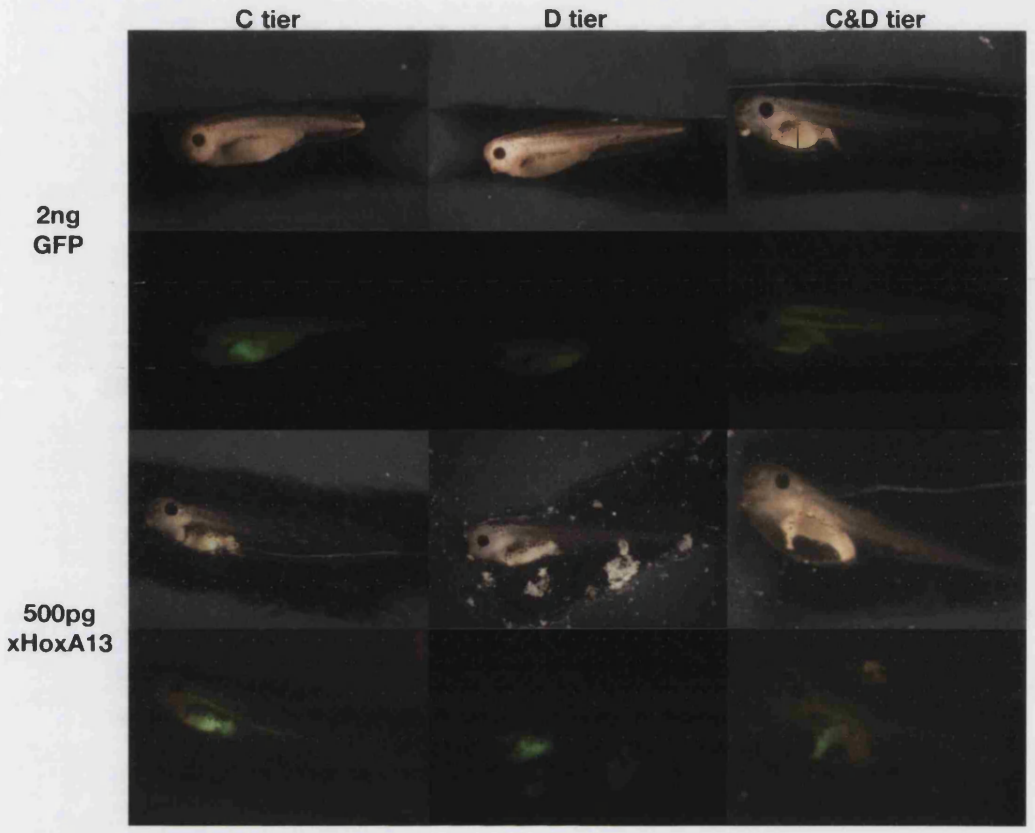


Figure 7.11 Injections of *GFP* and *xHoxA13* done on 32-cell stage embryos. Injections were done on the C3 and C4 or D3 and D4 or all four blastomeres with *GFP* and *xHoxA13*. Here we can see that *xHoxA13* seem to only induce malformations of the gut when injected on all of C3&4 and D3&4 blastomeres.

Earlier we have discussed that at 32-cell stage embryo, the C3 and C4 blastomere contribute mostly towards mesodermal cells and that the D3 and D4 blastomere contribute mostly towards endodermal cells. Thus if we coinjected *GFP* with the *xHoxA13* mRNA into C3 and C4 blastomere and coinjected rhodamine-dextran with *xHoxA13* mRNA into D3 and D4 cells then the mesodermal cells that have taken up the RNA should be labelled in green and the endodermal cells that have taken up the RNA should be labelled in red. This would allow us to study how the mesodermal and endodermal cells interact in the *xHoxA13* embryos. We decided to use *GFP* on the C3 and C4 as *GFP* is more sensitive than rhodamine-dextran. There are fewer mesodermal cells compared to endodermal thus to allow for better visualisation it is better to use the more sensitive label to indicate the less abundant mesoderm.

The *GFP* control was used as before, with 500pg coinjected with or without the *xHoxA13* mRNA into the C3 and C4 blastomeres. With the rhodamine-dextran 4.6nl at 100µg/ml was coinjected with or without *xHoxA13* into the D3 and D4 blastomeres. We did a series of injections and coinjected *xHoxA13* at C3 and C4 only, D3 and D4 only, and in all ventral vegetal blastomeres similar to the previous 32-cell injection. However unlike the previous 32-cell injections we started observing the phenotype in all of the injections, when previously only when all of C3, C4, D3 and D4 *xHoxA13* injections lead to a phenotype.

The reason for this difference becomes clear when we cryosectioned the embryos to identify where the labels have gone. Figure 7.12 shows photographs of an injection with *GFP* and rhodamine-dextran labels only without *xHoxA13* mRNA. Looking at the transverse sections we can clearly see that the green from the *GFP* and the red from rhodamine-dextran show significant overlap, especially in the endoderm. If we assume that the overlap was due to the signals diffusing out and then mixing then it is also possible that the *xHoxA13* may have also diffused similarly. This would then mean that the affected cells in a C3 and C4 or D3 and D4 injections might now mimic the affected cells seen in C3, C4, D3 and D4 injection leading to the apparently random appearance of the *xHoxA13* phenotype in this round of injections.

We believe that this overlap might be due to the molecules moving across the blastomere boundary during injection. The time needed to inject with two labels instead of one is greater. To account for this extra time needed, we had to start our injections earlier, just as the blastomeres of the 32-cell stage embryos are forming. By doing this, we might have injected the labels/mRNA into blastomeres that did not have a fully formed cell boundary yet. This in turn might have lead to the labels/mRNA diffusing between the two blastomeres leading to the overlap we see between the *GFP* and rhodamine-dextran.

Figure 7.12 32-cell stage injections with 2 fluorescent labels.
Injections were done on 32-cell stage embryos on the C & D tiers. Injections at C-tier was marked with *GFP* and injections at D-tier was marked with Rhodamine-dextran. A) Shows whole embryo photos of the location of the markers. B) shows photographs of transverse sections of the embryos injected with these two markers.

VII.4. Discussion

Our overexpression experiments shows that of the two most posterior *Hox* tested *xHoxA13* is most likely to be involved in gut development as *xHoxD13* overexpression did not have any visible effects on the gut development. In the *xHoxA13* injections, the embryos developed a bubble (oedema) around the gut. However when we performed *in situ* with these guts for *Xlhbox8* and *Xcad2* we found that the normal expression pattern of specification markers *Xlhbox8* and *Xcad2* was maintained. This persistence of the endoderm patterning was unexpected in view of the degree to which the gut has altered in shape. However, we need to remember that some of the endoderm was not affected as we did not inject the dorsal vegetal side. According to fate maps this blastomere might contribute to some of the *Xlhbox8* expressing region (Chalmers and Slack, 2000; Dale and Slack, 1987), and as such a lack of the *Hox* mRNA in this region could mask a posterior respecification as *Xlhbox8* would not be downregulated.

It might not be possible to investigate this possibility using RNA injection as injections to the dorsal vegetal side at the 8-cell stage might also induce some of the defects seen with the dorsal injections at the 4-cell stage, thus complicating the phenotype. Instead, to ensure expression in the entire endoderm without disrupting head structure it might be necessary to use a combination of transgenics and grafting. In this method, the *Hox* genes would be expressed throughout the whole embryo transgenically under the control of CMV promoter. Then around the gastrula stage , the endoderm and mesoderm from this transgenics embryo would be cut out and grafted to a donor embryo. Thus the recombinant embryo would just express the *Hox* genes in the whole endoderm and mesoderm, including the *Xlhbox8* expressing region, allowing for detection of posterior respecification.

Since there was no change in specification of the endoderm in the ventral vegetal injections we decided to focus on the visible morphological

changes in the gut. Observing the whole embryo there was an oedema that was formed around the gut. The gut itself contained a large cavity, lacked coiling and seemed to attach itself to the dorsal side of the embryo. We are currently unsure as to the role the oedema might play in the formation of the gut phenotype. There is a possibility that the formation of the oedema might have lead to the formation of the deformed gut. The water that is taken up inside the oedema might act as an obstacle to the endoderm, preventing its normal morphogenetic movements thus leading to the deformed gut observed. Thus far we have not been able to exclude this possibility as all the deformed gut observed thus far were always accompanied by the oedema. Hence at this point we can only conclude that the malformation of the gut and formation of oedema is somehow linked.

Looking at transverse sections of the *xHoxA13* injected embryos shows that the gut of the affected embryos to be distinctly different from the uninjected ones. We were able to confirm whole mount observations that the *xHoxA13* embryo guts did not show coiling. We were also able to discover that the guts of the *xHoxA13* embryos were not a solid mass of cells towards the posterior of the embryo. A cavity has formed in the middle of the endoderm in this part of the embryo. Also the layers of cells seem much thinner in the *xHoxA13* embryos, especially towards the dorsal side where the cells appear to be only 1-2 layers thick. We suggest that this may be due to disruption of the morphogenetic movements of the gut. Perhaps the *xHoxA13* has promoted some cell-cell intercalation that would lead to the thinner layer of cells as well as the cavity in the middle of the endoderm.

We also traced the location of the coinjected *GFP* labels on cryosectioned embryos and found that in the abnormal *xHoxA13* embryos, most of the mRNA was in the endoderm and not mesoderm. Initially we had thought that the *xHoxA13* was eliciting its effects in the endoderm, however subsequent experiments confining the *xHoxA13* to the endoderm only failed to generate the same phenotype. Injections of *xHoxA13* into D3 and D4 blastomeres of 32-cell stage embryo only resulted in normal embryos. It

was only when all the C3, C4, D3 and D4 blastomeres were injected that the *xHoxA13* phenotype was generated. This then indicated that the expression of *xHoxA13* in the mesoderm as well as the endoderm is necessary to generate the *xHoxA13* phenotype.

At the moment we believe that the key to understanding this phenotype is an ability to visualise and understand the relative movements of the mesoderm and endoderm in the *xHoxA13* embryos. We attempted to do this by injecting two different fluorescent dyes, *GFP* and rhodamine-dextran to the mesoderm and endoderm respectively. However we encountered a technical hurdle with this approach. Due to the extra time needed to inject both labels to the appropriate blastomeres, the injection had to be done earlier just as the blastomeres of the 32-cell stage embryo formed. This extra time would not be needed if we only had to inject a small number of embryos, but because of the incomplete penetrance of the *xHoxA13* phenotype it is important to inject at least 15 embryos per experiment to get a significant number of abnormal embryos. This, combined with the difficulty of obtaining regularly cleaving embryos makes it difficult to get a reasonable result with this method. To solve this problem we would need to develop a faster way of injecting the two labels into their respective blastomeres. Perhaps with 2 people doing the injections in tandem we might be able to do the injections later and still achieve a statistically significant number with the phenotype.

We still believe that this method is an appropriate approach to investigate the mechanism and causes of the *xHoxA13* phenotype. It is the most straightforward and simple way to deliver two separate labels to two separate groups of cells. We could in principle use transgenics to achieve this but this would mean that we need to make two separate constructs with two separate promoters, one for the endoderm and another for the mesoderm. This would take a much longer time than simply optimising the RNA injection method. Transgenics would probably be more useful at a later

stage in the investigation, once the mechanism is known to help with the more specific and delicate experiments.

In conclusion we found that *xHoxA13* is able to disrupt endoderm development on overexpression. It appears to perturb the morphogenesis of the gut but not the specification of the endoderm. The expression pattern of *Xlhbox8* and *Xcad2* seems to be more stable than previously expected, although this might be due to incomplete delivery of *Hox* RNA to the *Xlhbox8* region. Thus far we have been able to show that the malformations of the gut only appears when *xHoxA13* was expressed in both the mesoderm and endoderm. However we need to remember that an overexpression experiment such as this does not prove involvement of *xHoxA13* in the normal development of the endoderm. To do this we would need to perform a loss of function experiment of some sort. Perhaps by using morpholinos to knock down the levels of *xHoxA13* mRNA in the embryos.

VIII. Discussion

VIII.1. Endoderm specification – current model

Regional specification of the endoderm is defined as the commitment of each tissue region, which is manifested on culture in a neutral medium but may still be reversible by grafting to a new position (Slack, 1983). Like other embryological concepts, this one dates from the pre-molecular era. Nowadays, in *Xenopus* , we consider it is possible to observe specification by the expression of transcription factors such as *Xlhbox8* and *Xcad2* that would later drive the expression of tissue specific genes in fore and mid-hind gut respectively (van den Akker et al., 2002; Wright et al., 1988). There may also be advanced activation of some differentiation products such as *IFABP* during this stage of development (Shi and Hayes, 1994). Throughout the years there have been two conflicting models for the specification of the endoderm in *Xenopus*. The early model based on vegetal explants (Gamer and Wright, 1995; Wright et al., 1988; Zorn et al., 1999) and the late model based on stage 20-23 endoderm explants (Horb and Slack, 2001).

The early model proposed that the specification of the endoderm occurs early in development (prior to gastrulation), cell-autonomously and in the absence of mesoderm. Certain regions of vegetal explants from blastula stage embryos, was shown to express *Xlhbox8* and *IFABP* (Gamer and Wright, 1995; Wright et al., 1988). Anterior endoderm in particular was thought to be specified by the early blastula stage as marked by the expression of *Xhex* and *Cerberus* (Zorn et al., 1999). However recently this apparent cell-autonomous specification was shown probably to be due to previously undetected presence of mesoderm (Horb and Slack, 2001).

Indeed it was shown by these authors that endoderm-only explants made from a later stage embryo (stage 20-23), where the endoderm and mesoderm can easily be separated, does not express *Xlhbox8* or *Xcad2,* indicating lack of specification. The presence of mesoderm is essential for

the initial and continued expression of both *Xlhbox8* and *Xcad2*. Heterologous recombinations done by Horb and Slack, also indicated that the signals sent by the mesoderm is acting in an instructive manner. Anterior endoderm took on a posterior specification when recombined with posterior mesoderm. Similarly posterior endoderm takes on an anterior specification when recombined with anterior mesoderm. This then lead to the second model of endoderm specification where its specification took place later in development (neurula stages) and was dependant on instructive signals from the mesoderm (Horb and Slack, 2001).

In our study, we have made findings that both agree and disagree with the Horb and Slack model. Similar to Horb and Slack (2001) we found that mesoderm was indeed essential for the specification of the endoderm as isolated endoderm failed to express both *Xlhbox8* and *Xcad2*. This also confirms that the endoderm specification occurs late in development and is not cell-autnonomously. However unlike the earlier Horb and Slack study (2001) we found that the mesoderm was not capable of instructing the mesodermal fate. In our recombinations the endoderm maintained its original fate regardless of whether or not it was recombined with anterior or posterior mesoderm. We have considered several reasons for why we observed a permissive instead of an instructive one.

Out of the different possibilities (see Chapter 5 Discussion, p100) we believe that the discrepancy in results is probably due to the variable nature of the explants. The cuts done to create the explants are somewhat arbitrary and as such the results themselves can be variable if one is not careful. Thus, it was possible that Horb and Slack in their study did not perform enough repetitions to see this variability. Also, no in situ data was obtained alongside their PCR to confirm that the mesoderm was instructive. Considering that we obtained at least three consistent and repeatable with with our recombinations with both *in situ* and PCR, we believe that our result is the correct one and that the mesoderm is permissive not instructive at stage 20.

This discovery meant that the endoderm must have been patterned earlier, and that up to the mid 20s stages continued association with mesoderm is necessary to maintain it. It is currently unclear as to how or when it receives its patterning as our attempts to make endoderm-only explants from early embryos (stage 10.5 and 13)) were unsucccessful: the explants always contained some mesoderm. However previous studies have found that *Xlhbox8* is expressed as early as stage 12.5 (Wright et al., 1988). Thus, it is conceivable that the endoderm is also patterned around this stage. This pattern is then maintained by the mesoderm, becoming stable after stage 25, presumably when the endoderm and mesoderm stop their relative movements and become finally aligned with each other (Chalmers and Slack, 2000). According to the traditional definition the period of mesoderm-dependent commitment is not specification since it is not maintained in a neutral medium. However it is specification if endoderm and mesoderm are considered together as a unit.

The fact that the mesoderm is permissive rather than instructive also had implications for our screens. Initially we wanted to screen various growth factors and inhibitors on endoderm-only and endo+meso explants in an attempt to identify the mesodermal signals. Any of these screens should be able to respond to an instructive signal, so long as the competence of the endoderm to respond is still maintained at the stage of testing. However if the mesoderm is permissive, then the growth factor screen on endo+meso explants becomes redundant. The endoderm in these explants already shows the normal anterior expression of *Xlhbox8* and posterior expression of *Xcad2*. This normal expression would not change in the presence of any growth factors that are mimicking a permissive mesodermal signal.

The growth factors on endoderm-only explant, however, would still be relevant. In this screen the endoderm-only has no expression of *Xlhbox8* or *Xcad2*, thus a growth factor mimicking a permissive signal should show recovery of the anterior expression of *Xlhbox8* and posterior expression of *Xcad2*. Similarly the inhibitor screen on endo+meso explants would still be

relevant as molecules that inhibit specification would result in a downregulation of the normal expression domain of *Xlhbox8* and *Xcad2*.

Thus based on this we can still confirm that neither Activin A, BMP4, FGF4, FGF8, FGF10, EGF, or RA was able to induce specification in stage 20 endoderm-only explants. We also excluded a possible role for Wnt as LiCl treatment of explants, which mimics Wnt activation by inhibiting GSK-3β, did not yield any change to expression pattern of *Xlhbox8* and *Xcad2*. This was consistent with the inhibitor screen as inhibitors of these pathways were shown to not affect the expression domains of *Xlhbox8* and *Xcad2*.

VIII.2. Future work on endoderm specification

From our results we believe there are two main areas of interest that would need addressing to better understand the specification of endoderm in *Xenopus*. The first would be the mesodermal signals at stage 20. We have shown that even though they ae not capable of respecifying the endoderm they are still essential for the maintenance of expression of *Xlhbox8* and *Xcad2*. It would be interesting to see how this is done, we have tested several growth factors and have excluded them from having a role in endoderm specification, at least as single agents.

However this list of growth factors was not exhaustive as it is limited by commercial availability. We have discussed previously testing for newly available commercial growth factors as well as possible new and novel growth factors that came out of the screen of anterior endoderm carried out by Aaron Zorn (see chapter 4 discussion, p84). Also we need to test for the possibility that the growth factors are acting collaboratively and not as a single inductive signal. We can directly test collaborative signals as well as newly available commercial factors using the endoderm explant method.

With commercially unavailable as well as novel factors we could perhaps use an oocyte based system to express them. Manually defolliculated oocytes when injected with mRNA coding for the protein of interest continually produce and secrete that protein when cultured in oocyte

culture medium (OCM). This method has been used previously with animal caps to successfully reconstitute inductive signaling (Lustig and Kirschner, 1995). This test could then be adapted to study endoderm respecification by putting the endoderm or endo+meso explants on the injected oocytes and thus allowing the expressed protein to affect the explants.

The other area of interest would be the earlier endoderm patterning event. We showed that stage 20 mesoderm is only maintaining a previously existing pattern. Our attempts to study this possible earlier patterning event using stage 10.5 and 13 embryos were unsuccessful. We found that, similar to vegetal explants made from stage 9 embryos, endoderm explants made from stage 10.5 and 13 showed a significant amount of *xFOG* and *FoxF1* indicating the presence of mesoderm. Expression of these two markers was noticeably missing from stage 20 endoderm-only explant. This meant that we could not perform a growth factor screen using earlier endoderm explants.

Thus to study the earlier patterning event we need to consider new methods that do not require generation of endoderm-only explants. Recently there was introduced a new method that allows for selective ablation of cells. This uses a modified viral protein known as M2(H37A), which is a small integral membrane protein isolated from a variant of the influenza virus. Normally this protein functions as a proton selective channel of the trans Golgi compartment during influenza infection. However if the protein is modified to have a His-Ala subtitution at the 37^{th} residue the protein acquires a much broader specificity for all cations (Chizhmakov et al., 1996; Ogden et al., 1999; Tang et al., 2002). This in turn makes the protein much more toxic and it has been shown to be capable of killing transfected mammalian cells unless they are cultured with the anti antiinfluenza drug rimantadine (Smith et al., 2002). This is because rimantadine binds to the channel protein, preventing its function and consequently its toxicity.

In *Xenopus* it was demonstrated that microinjection of M2(H37A) RNA into selected blastomeres of early stage embryos results in death of their progeny by late blastula stages (Smith and Mohun, 2004). This meant that the protein is toxic as well in *Xenopus*. However if these injected *Xenopus* were cultured in medium containing the drug rimantadine, then they developed normally. This then means that the method enables temporal control as to when the cells are killed. This is important in our study of endoderm specification as this would allow us to ablate the mesodermal cells just before regional specification occurs and not disturb the earlier development events.

To ablate mesodermal cells specifically we would also need spatial control for the expression of the M2(H37A) protein. A recent paper describes the isolation of the 5' promoter region for *FoxF1*, a gene expressed almost exclusively in the mesoderm around the endoderm (Tseng et al., 2004). This promoter has been shown to successfully drive expression of *GFP* and *LacZ* specifically in the lateral plate mesoderm. Hence it follows that if we put the gene M2(H37A) protein under the control of the *FoxF1* promoter we would be able to isolate the expression of the channel proteins to the mesoderm.

Thus the complete method for ablating the mesoderm around the endoderm would involve transgenically expressing the M2(H37A) protein under the control of the *FoxF1* promoter. The transgenic embryo is then grown in medium containing rimantadine until it reaches stage 20 where the drug is removed, activating the channel protein and selectively killing the mesodermal cells. Various different growth factors attached to heparin-acrylic beads could then be implanted in these mesoderm free embryos, which should now be suitable for detecting permissive growth factors allowing for further study into the role of mesoderm at this particular stage of development.

We need to note however that this method is still just an idea and would require a good deal of optimization. Also we need to note that we

were ourselves unable to obtain a working transgenic with the *FoxF1* promoter. Discussions with members of Aaron Zorn's lab revealed that other labs are having problems with this particular promoter as well. Despite all of these drawbacks, it might be worth pursuing this method as it could create an elegant *in vivo* system to study regional specification in *Xenopus*.

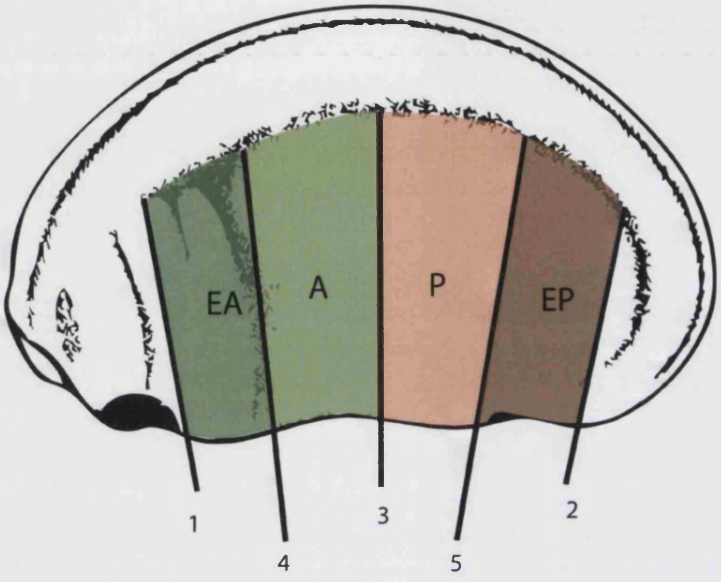


Figure 8.1 Stage 20 quarter explants.
Diagram above shows how the stage 20 embryo is divided in to four quarters from anterior to posterior: extreme anterior (EA), anterior (A), posterior (P) and extreme posterior (EP).

Other than these two major areas, we would also like to follow up a particularly intriguing result we observed during quarter explant heterologous recombination. The downregulation of *Xcad2* when the extreme anterior endoderm were recombined with extreme posterior mesoderm. This downregulation of the posterior marker of development by posterior mesoderm was puzzling. We were not able to come up with a satisfactory model to explain this with our current ideas. Initially we thought that this could indicate that *Xcad2* expression at the extreme anterior endoderm is very labile or that the *Xcad2* inducing signal is coming from the middle of the embryo. However both these model does not explain why the *Xcad2* expression is maintained with extreme anterior mesoderm recombination. At the moment more data is needed, more heterologous

recombinations needs to be done with the other two regions in the middle of the embryo (A and P in Figure 8.1). This is to confirm whether this downregulation is specific to the extreme posterior mesoderm or occurs with any mesoderm except the extreme anterior mesoderm.

VIII.3. *Hox* Genes expression in Xenopus mesoderm

In our study we also attempted to investigate how the mesoderm obtained its positional identity so that it can subsequently pattern the endoderm. The *Hox* genes were our main focus for this investigation. In chick the *Hox* genes have been found to be expressed in a nested, overlapping patterns in the developing gut mesoderm (Roberts et al., 1995; Sakiyama et al., 2000; Sakiyama et al., 2001; Yokouchi et al., 1995). Interestingly the boundaries of expression pattern of some of these mesodermal *Hox* genes align with the morphological borders of the different gut regions (Grapin-Botton, 2005).

Similar nested expression pattern have been seen with the *Abdominal B*-type *Hox* in Xenopus (Lombardo and Slack, 2001). In this study *xHoxA9, xHoxD9, xHoxD10, xHoxC12* and xHox*A13* were shown to be regionally expressed in the mesoderm. These genes were also found to be expressed in isolated gut of stage 42 embryos with *xHoxA13* thought to be expressed in both mesoderm and endoderm. Our study builds on this and tested for the expression of these *Hox* genes as well as *xHoxD13* in endoderm-only and endo+meso explants. Through *in situ* hybridisation we have shown that *xHoxD9, xHoxD10* and *xHoxC12* are expressed in the endo+meso explants. Since endo+meso explants contains the minimum amount of tissue for the correct specification of the endoderm, this then is consistent with a possible role for *Hox* genes in endoderm specification.

xHoxA13 and *xHoxD13* was found to be expressed in a modified endo+meso explant that included more posterior regions. Because of this modification we were not able to use similar arguments to support their possible role in endoderm specification. Their posterior expression indicates

that they might be expressed at the proctodeal level, a different territory from the future intestine (Chalmers and Slack, 2000). However, it is still possible that *xHoxA13 and xHoxD13* might still be involved in endoderm development. It is conceivable that *xHoxA13 and xHoxD13* can induce the formation of a posterior gradient from the proctodeal region which in turn will pattern the endoderm.

We did not obtain any clear staining with *xHoxA9* in endoderm-only, endo+meso explants or isolated stage 42 gut. However the *in situ* on stage 35 whole embryos showed the expected expression pattern for xHoxA9. We believe that the lack of staining with the *xHoxA9* probe on the explants and gut represents a downregulation in the expression of *xHoxA9* by stage 42.

We were also able to resolve the germ layers at which these *Abdominal B*-type *Hox* is expressed, the endoderm and mesoderm. By sectioning gut following wholemount *in situ* we found that *xHoxD9, xHoxD10, xHoxC12* and xHox*D13* are expressed in the mesoderm. *xHoxA13* was also expressed in the mesoderm with a possible gradient expression towards the endoderm. We have discussed several possible reasons for the graduated expression of *xHoxA13,* including an *FGF8* like RNA gradient (Delfini et al., 2005; Dubrulle and Pourquie, 2004) or it might represent a different threshold of *xHoxA13* activation in endoderm compared to mesoderm. To determine which might be correct we believe that it is necessary to investigate activation of the downstream molecules of *xHoxA13* as well as its normal function in *Xenopus*. This would probably best done using either differential RNA analysis or microarrays.

VIII.4. *Hox* overexpression and endoderm

We also investigated the possible functions of *Hox* genes in endoderm specification by overexpressing the two most posterior *Hox xHoxA13 and D13* through RNA injections. Previously in chick it was demonstrated that loss of expression of *Hoxa-13* and *Hoxd-13* results in the

alteration of muscle layers of the sphincter, which is consistent with a partial anterior transformation of this region (Kondo et al., 1996; Warot et al., 1997).

In our study we found no abnormal phenotype with *xHoxD13* injections. The expression domains of *Xlhbox8* and *Xcad2* was similar to control uninjected embryos. However we did see an altered phenotype with the *xHoxA13* RNA overexpression. We found that an injection to the vegetal ventral side of 8-cell stage embryo induced the formation of an oedema and affected the morphogenesis of the gut.

We were surprised to find that the normal expression domains of *Xlhbox8* and *Xcad2* were maintained in these deformed guts. However we later discovered that this lack of respecification could be due to a technicality. At the time of the experiment we decided to deliver the RNA to most of the gut by injecting to the ventral vegetal blastomeres of 8-cell stage embryo. We avoided injecting to the dorsal vegetal blastomeres because there was a possibility of inducing a head/anterior suppression which would complicate the analysis of the results. However in doing this we might have inadvertently not delivered fully the *Hox* RNA to the *Xlhbox8* expressing region in the embryo. According to the fate map dorsal vegetal blastomere can contribute to some of the anterior dorsal and ventral regions that form the *Xlhbox8* domain (Chalmers and Slack, 2000; Dale and Slack, 1987). As such the incomplete delivery of the *Hox* RNA to the *Xlhbox8* expressing region might have prevented its dowregulation, making it impossible to detect posterior respecification of the endoderm.

We also attempted to further characterise the obvious deformities seen in the gut of *xHoxA13* injected embryos. Sections revealed a cavity developing in the gut endoderm. We believe that this cavity is due to abnormal cell-cell intercalation movements rather than cell death as we did not observe any necrotic cells in the gut. We also found that the abnormal phenotype became visible around stage 39 and 41. However because we did not control the temporal expression of the *xHoxA13* RNA we cannot rule

out the possibility that the *xHoxA13* might be affecting earlier development and that the effect only manifests itself by stage 39.

By observing the localisation of coinjected GFP we were able to identify that with the abnormal phenotype, the *xHoxA13* RNA was mainly in the endoderm. However we believe that this is not due to more endodermal precursors taking up the RNA during the injections. Our injections to the D3 and D4 blastomeres at 32-cell stage, which would have affected mostly endodermal cells, could not induce the phenotype. Instead we found that injections to the C3 and C4 blastomeres, which contributes to mostly mesodermal cells, alongside injections to D3 and D4, was necessary to induce the phenotype. This then indicates that uptake of the *xHoxA13* RNA by the mesodermal cells is necessary to induce the phenotype. Thus the earlier observation of GFP in endoderm only could be due to either the mesodermal cells that took up the RNA moving towards the endoderm and disrupting the morphogenesis or that the mesodermal cells themselves were dying off and as a consequence causing disruption to the morphogenesis of the gut.

VIII.5. Future work on *Hox* genes in Xenopus

With the Hox experiments there are several things that need to be addressed. The first is to correct the overexpression of the Hox genes to overexpress them fully in the *Xlhbox8* region to allow for detection of posterior respecification. We feel that for this purpose RNA injections would probably not be the best approach as the dorsal-vegetal injection necessary to do this would also induce head/anterior structure suppression complicating the analysis. Instead we should consider using a combination of transgenics and grafting. First the *Hox* genes would be expressed throughout the whole embryo under the control of a global promoter such as CMV. Then around gastrula, the endoderm and mesoderm from the transgenic embryo would be grafted to a host embryo. Thus the recombinant embryo would just express the Hox genes in the whole of endoderm and mesoderm, including the *Xlhbox8* expressing region.

We also still need to investigate why *xHoxA13* expression was only found in the endoderm in the abnormal phenotype when it needs to be overexpessed in both mesoderm and endoderm. We believe that the best way to study this is still by using two fluorescent markers. This would help visualise how the endoderm and mesoderm move with respect to each other and give insight into how their interaction causes the abnormal phenotype. At the moment the biggest problem with this method is speed, we could not do it quickly enough to avoid the problem of RNA leaking from one blastomere to another. However this can be easily avoided by either involving two people to do the injections and cut the time in half or by somehow optimising the injection method to make it faster.

There are also other Hox genes, *xHoxD9, xHoxD10* or *xHoxC12,* that have been shown to be expressed in the mesoderm but have not yet been tested for possible involvement in endoderm specification. The transgenic and grafting method described above should be suitable for overexpressing these *Hox* genes to see if they would disturb the specification of the endoderm.

Finally we must also remember that thus far we have only considered overexpressing the *Hox* genes. Overexpression itself is not enough to conclusively say that a particular *Hox* is involved in the normal development mechanism. For this purpose it is essential that we do a complementary knockout study alongside the overexpression. This would probably be done using morpholinos to knock down the RNA levels of the *Hox* in the embryos and observing if it could disturb the specification of the endoderm.

VIII.6. Conclusion

Our results supports, in part, the Horb and Slack model of endoderm specification. We also found that the endoderm does not become specified until after stage 25 and that the presence of mesoderm is essential for specification of the endoderm. However, contrary to Horb and Slack (2001), we believe the mesoderm between stage 20-25 is not capable of directing

endoderm specification, instead it is only maintaining a previously existing pattern. We have also shown that the mesodermal signals maintaining expression of *Xlhbox8* and *Xcad2* between stage 20-25 is not Activin A, BMP4, FGF4, FGF8, FGF10, EGF or RA. We are also confident that the mesodermal signals do not belong to the WNT signalling pathway.

Obviously this model of endoderm specifcation in *Xenopus* is still incomplete. We still have not discovered the identity of the permissive mesodermal signals at stage 20. There are new novel factors, recently isolated from a microarray on anterior endoderm (Aaron Zorn, unpublished data), that need to be tested for possible involvement in endoderm specification. We are also still unsure when the initial patterning event takes place, although looking at the temporal expression of *Xlhbox8* it might be as early as stage 12.5 (Wright et al., 1988).

Thus far based on our results we are still unable to either prove or disprove possible roles of *Hox* genes in endoderm specification. We have shown that they are expressed in the correct germ layer to provide positional information to the mesoderm which would in turn allow it to pattern the endoderm. However because of a technical limitations, RNA overexpression of *xHoxA13* and *xHoxD13* might not have affected the *Xlhbox8* region, thus masking the possible posterior respecification of the endoderm. Although we did obtain an interesting gut deformation phenotype that is induced by overexpression of *xHoxA13*, showing that it disrupts the morphogenesis of the gut. We have not completed our characterisation of this phenotype but initial analysis indicates that overexpression needs to be in both mesoderm and endoderm and that the phenotype might be due to disruption of cell-cell movement.

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